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Review

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Eight Kinetically Stable but Thermodynamically Activated Molecules that Power Cell Metabolism

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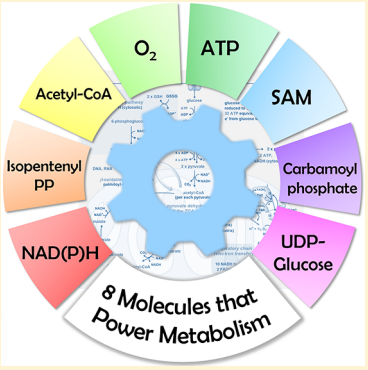
ABSTRACT: Contemporary analyses of cell metabolism have called out three metabolites: ATP, NADH, and acetyl-CoA, as sentinel molecules whose accumulation represent much of the purpose of the catabolic arms of metabolism and then drive many anabolic pathways. Such analyses largely leave out how and why ATP, NADH, and acetyl-CoA (Figure 1) at the molecular level play such central roles. Yet, without those insights into why cells accumulate them and how the enabling properties of these key metabolites power much of cell metabolism, the underlying molecular logic remains mysterious. Four other metabolites, *S*-adenosylmethionine, carbamoyl phosphate, UDP-glucose, and Δ^2-isopentenyl-PP play similar roles in using group transfer chemistry to drive otherwise unfavorable biosynthetic equilibria. This review provides the underlying chemical logic to remind how these seven key molecules function as mobile packets of cellular currencies for phosphoryl transfers (ATP), acyl transfers (acetyl-CoA, carbamoyl-P), methyl transfers (SAM), prenyl transfers (IPP), glucosyl transfers (UDP-glucose), and electron and ADP-ribosyl transfers (NAD(P)H/NAD(P)$^+$) to drive metabolic transformations in and across most primary pathways. The eighth key metabolite is molecular oxygen (O_2), thermodynamically activated for reduction by one electron path, leaving it kinetically stable to the vast majority of organic cellular metabolites.



CONTENTS

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Chem. Rev. 2018, 118, 1460−1494

1. INTRODUCTION

Delineation of metabolic pathways, the tandem conversion of one cellular small molecule (MW < 1500 Da) to the next, from initial entry point to final product, each termed a metabolite, were the tasks of two to three generations of biochemists and molecular physiologists during the first half of the twentieth century. Because metabolites are by and large stable small molecules, they were most readily accessible to purification and structural characterization compared to biological macromolecules such as oligosaccharides, proteins, and both RNA and DNA. As the pathways for some 8000 (see human metabolome database, release 3.0[1]) stable small molecule metabolites became filled in, there were parallel efforts to characterize the high molecular weight protein catalysts, enzymes, that carry out the separate steps in each metabolic pathway.

Primary sequence determination and three-dimensional structural elucidation of the macromolecular biomolecule classes, proteins, RNA, DNA, and oligosaccharides, consumed much of the focus of the second half of the twentieth century. That structural work went hand in hand with invention of advances in protein and nucleic acid crystallographic methods as well as high-resolution solution techniques in NMR and mass spectrometry. The recent advent of systems biology approaches has led to the hierarchy of omics platforms.[2] Integration of genomics, transcriptomics, proteomics, and metabolomics to connect any or all of these system-wide changes to phenotype is now a dominant contemporary paradigm in cell biology.

In particular, the ability to measure simultaneously hundreds of small molecule cellular metabolites by mass spectrometry in many cell types and under many perturbation conditions has generated the subfield of cell metabolism as a window into the moment-to-moment biology of cells, tissues, and organs.[3] In turn, this has sparked a renaissance of interest in how metabolic pathways function and interact, including how they are linked to the plethora of parallel, orthogonal, and converging signaling inputs from temporal and spatial regulation of enzymes in various pathways. The simultaneous measure of many metabolites at once (metabolomics) promises to give new insights into the physiology of cells and tissues.

At first glance, maps of metabolic pathways pose stultifying complexity. They are so data-rich that they are unreadable except in highly abbreviated form where molecules are not represented with chemical structures or even cartoons but typically one word descriptors. With more than eight-thousand known metabolites of MW < 1500 Da and hundreds of enzymes catalyzing their interconversions, dozens of pathway nodal points, and the existence of several layers of regulation at key points (such as gatekeeper enzymes and rate-limiting enzymes), the challenge of full integration and predicting cellular responses remains complex and yet unrealized.

1.1. Seven Molecules with Group Transfer Potential

We argue in this review that much of the intertwined logic of metabolic pathways (e.g., in human and other higher eukaryotic cells) can be understood by focus on seven molecules whose structures enable a diverse set of group transfer reactions (Figure 1).[4] Three of these molecules, ATP, NADH, and acetyl-CoA are prominently accumulated in catabolic (degradative) metabolism and then used to drive anabolic small molecule and macromolecule pathways, including protein, nucleic acid, and oligosaccharide biosynthetic metabolism. Four others, *S*-adenosylmethionine (SAM), UDP-glucose, carbamoyl phosphate, and Δ^2-isopentenyl-PP, are additional key building blocks in biosynthetic pathways, also by virtue of group-transfer chemistry. An eighth, inorganic molecule, molecular oxygen, is the *ur*-electron acceptor in respiratory chains but is otherwise substantially restricted in its metabolic roles in higher eukaryotes. Of course, O_2 is not a functional energy source in anaerobic organisms.

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Chem. Rev. 2018, 118, 1460−1494

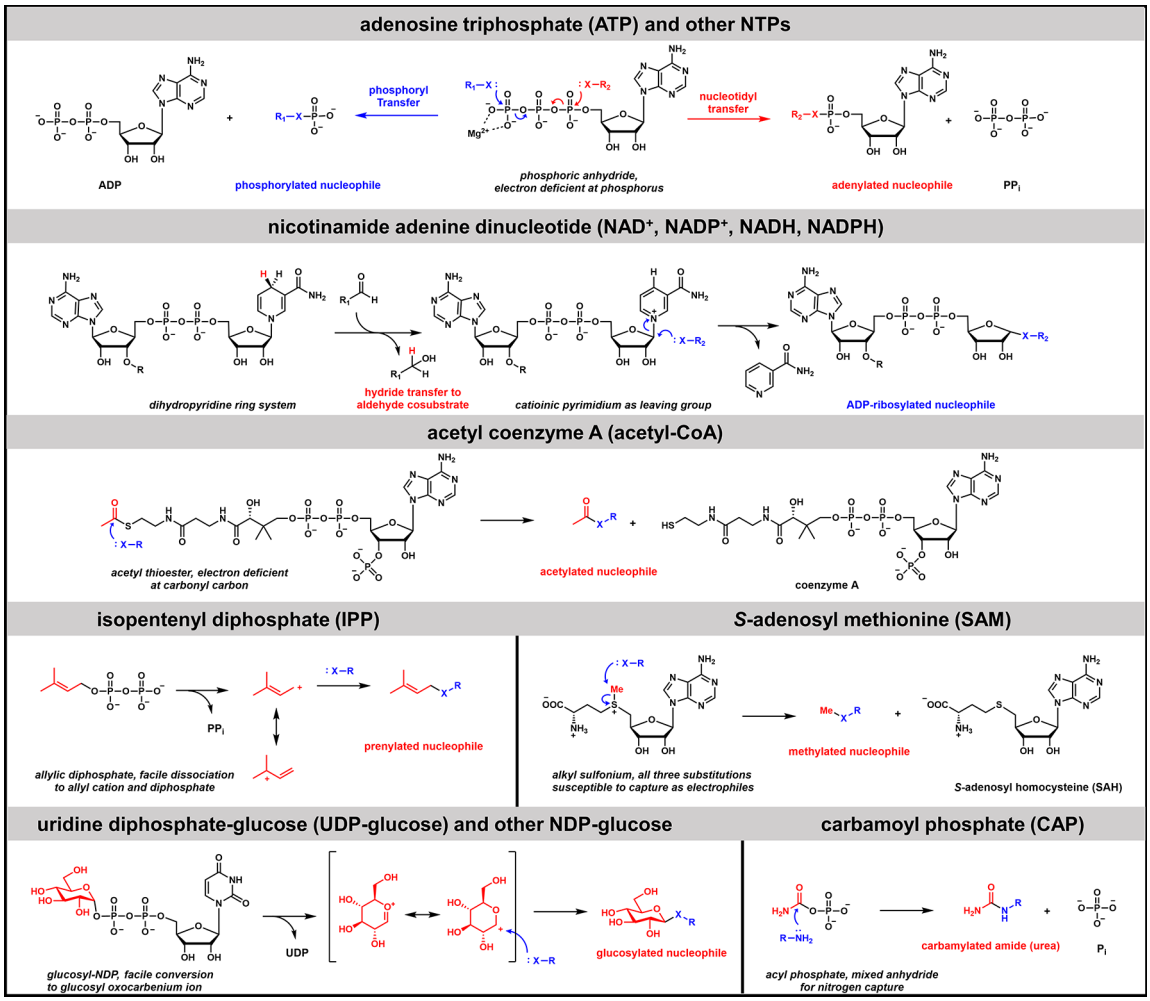

Figure 1. Seven organic molecules that power cell metabolism: ATP and related nucleoside triphosphates (NTPs); oxidized (NAD^+, $NADP^+$) and reduced (NADH, NADPH) nicotinamide adenine dinucleotides; acetyl-coenzyme A; Δ^2-isopentenyl diphosphate [also known historically as isopentenyl pyrophosphate (IPP)]; *S*-adenosyl methionine (SAM); uridine-diphosphoglucose (UDP-glucose) and related NDP-hexoses; and carbamoyl phosphate (CAP).

ATP, NADH, and acetyl-CoA are called out as central in every analysis of the end points of cellular energy metabolism.[5,6] The reasons these chemicals are so versatile and universally valued lies in their ability to act as phosphoryl or nucleotidyl donors (ATP),[7] acyl donors for building up biosynthetic carbon scaffolds (acetyl-CoA), and electron donors (NADH, NADPH) most notably to respiratory chain components on the way down to molecular oxygen. These three metabolites have the common capacity for thermodynamically favored group transfer potential to drive otherwise unfavorable reactions and allow accumulation of useful quantities of materials far away from equilibrium (e.g., DNA, RNA, proteins, and oligosaccharides). Figure 2a illustrates how a generic coupling of metabolites X and Y to X-Y in which the desired X-Y might only accumulate to one part in a hundred, can be driven to 999 parts in a thousand by coupled cleavage of ATP (the mechanisms may often involve X-PO_3 or Y-PO_3 intermediates[8]).

Four additional molecules whose physiological roles are tied to comparable group transfer potential are SAM, carbamoyl phosphate, Δ^2-isopentenyl-PP (IPP), and UDP-glucose (and congeners). SAM is the source of essentially all methyl groups transferred in cells and that can number in the millions of events per cell cycle. This is formally a transfer of an electron deficient C_1 *alkyl group* to cosubstrate nucleophilic O, N, S, and C atoms (Figure 1).[9] In analogy, the IPP regioisomer, while looking nothing like SAM structurally, is a facile source of a resonance-stabilized five carbon allyl cation. The iterated head-to-tail condensation Δ^2-IPP with its Δ^3-regioisomer is the chemical reaction manifold to build isoprenoid scaffolds both on the way to thousands of steroid metabolites and to polyprenyl coenzyme Q and dolichol-P derivatives.

UDP-glucose is the entry point metabolite for thousands of sugar residues that are incorporated in anabolic pathways into oligosaccharides, for structural (cellulose), defense (bacterial lipopolysaccharide), and energy storage (glycogen, starch) purposes.[9] UDP-glucose and related NDP-hexoses are likewise the building blocks for all *N*-linked and *O*-linked carbohydrate chains in the major forms of glycoproteins. The transfer is always of a glycosyl ring that is electron deficient at C_1 (as an oxocarbenium ion equivalent).

Carbamoyl-P is more restricted in its physiologic roles, sequestered in two arms of nitrogen metabolism but exemplifies the acyl transfer chemical potential built into the mixed anhydride scaffold. Most notably, carbamoyl-P is a metabolic device for capturing free NH_3 and then directing it either to

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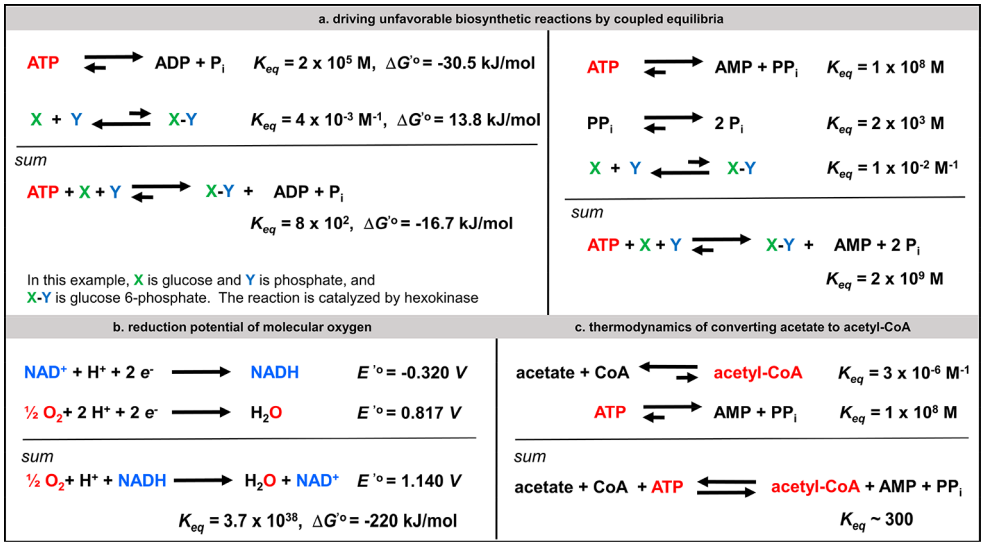

Figure 2. Thermodynamics of coupled metabolic reactions: (a) driving unfavorable biosynthetic reactions by coupled equilibria involving phosphoryl or adenylyl (nucleotidtyl) transfers that fragment ATP; (b) the favorable thermodynamics for four electron reduction of O_2 to 2 H_2O from 2 NADH is revealed by the reduction potential difference. This equilibrium is silent about the kinetic barriers to reduction; and (c) thermodynamics of coupled activation of acetate to acetyl-CoA as ATP is cleaved.

pyrimidine biosynthesis or to safe excretion as unreactive urea by action of a pair of carbamoyl transferase enzymes.[10]

Four of the seven molecules in Figure 1 are derivatives of adenine [ATP, NAD(P)H, acetyl-CoA, and SAM], while the fifth is a derivative of the UTP pyrimidine riboside (UDP-glucose). Carbamoyl-P arises directly from reaction of carbamic acid with ATP. This could reflect that they are all remnants of an RNA world[11] and remain current as useful fragments for both recognition and chemistry. The recognition could be that adenosine/adenine/AMP- and uracil/uridine/UMP/UDP-binding proteins evolved early in transition from an RNA world to a protein world. Such recognition domains might have been preconcentration devices for enrichment of adenine and uridine derivatives (high local concentrations in protein binding sites would favor reaction with additional reactants) that became CoASH, NADH, and SAM or progenitors thereof. In their current forms, the adenine and uracil moieties are not in the business ends of any of these five key metabolites. The distinct group transfer capacities have come into play as the other parts of the molecular scaffolds evolved (e.g the dihydronicotinamide ring in NAD(P)H, the sulfonium side chain of SAM, the thiol arm in CoASH).

The seventh metabolite, isopentenyl-PP, lacks any nucleoside moieties and is assembled in a short pathway from three molecules of acetyl-CoA that are condensed to the branched six carbon hydroxymethylglutaryl-CoA. Double reduction of the thioester to the alcohol gives mevalonate and then three ATP molecules are utilized (see section 7 and Figure 7). Two of these are for kinase-mediated phosphoryl transfer to the -PP linkage at C_1 and the third to give transiently the phosphate ester of the tertiary alcohol which eliminates to generate the key olefinic linkage in Δ^3-IPP. Subsequent isomerization of the olefin to the 2-position gives an equilibrium mixture of the Δ^2-IPP, and the characteristic prenyl chain pathway elongations can begin. As such, IPP isomers come directly from three acetyl group equivalents. In the C_{27} major sterol cholesterol the remains of 18 acetyl groups from acetyl-CoA can be spotted.

All five of these molecules require ATP or equivalent nucleotide triphosphate consumption for their assembly, stressing the energy expenditure for their accumulation. Coenzyme A and NAD^+ require both separate phosphoryl (-PO_3) and adenylyl (-AMP) transfers in their biosynthetic pathways. We noted just above that three ATPs are consumed on the way to IPP isoforms. SAM is sufficiently uphill from ATP that the two phosphoric anhydride linkages are cleaved during SAM synthetase action. Carbamoyl-P has a mixed acyl-phosphoric anhydride linkage as the business end of the molecule. The seventh key metabolite UDP-glucose is formed via uridylyl transfer from UTP.

In sum, a small number of group transfer reactions embedded within seven diffusable, kinetically stable but thermodynamically activated metabolites suffice to power most primary metabolic pathways. This is equivalent to stating that parsimonious chemical logic underlies the contemporary metabolic diversity (~2000−8000 known low molecular weight

Table 1

metabolic pathways	key substrates
glycolysis	ATP, NADH
pentose phosphate	NADPH, D-ribose-5-P, erythrose-4-P
glycogen assembly	UDP-glucose
glycoproteins	UDP-GlcNAc, GDP-mannose, UDP-galactose, UDP-glucose
tricarboxylate cycle	acetyl-CoA, NADH, GTP, Enz-$FADH_2$
fatty acid biosynthesis	ATP, acetyl-CoA, malonyl-CoA, NADPH, Enz-$FMNH_2$
fatty acid β-oxidation	long chain acyl-CoAs, acetyl-CoA, NAD^+, Enz-FAD
purine and pyrimidine biosynthesis	5-P-ribose-1-PP, E-FAD, ATP, carbamoyl-P
steroidogenesis	acetyl-CoA, IPP isomers
urea cycle	ATP, carbamoyl-P
Gln, Glu, α-ketoglutarate	NAD^+, ATP
phospholipid biosynthesis	SAM
nitrogen metabolism	glutamine, glutamate, NAD^+
protein PTMs	ATP, SAM, NAD^+, acetyl-CoA, FPP and GGPP, UDP-glucose, UDP-GlcNAc

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metabolites) in both prokaryotic and eukaryotic cells. Much of catabolic metabolism builds ATP, NADH (which builds up when respiration rates are low; e.g., low pO_2/hypoxia), and acetyl-CoA (both in mitochondria and cytoplasm) and these molecules can straightforwardly be converted to make SAM, IPP, and UTP (for UDP-glucose) and yield the master set.

Each of the seven molecules detailed in this section employ a distinct structural motif to provide a thermodynamically activated, kinetically stable framework that can be unlocked by metabolic enzymes to do useful cellular work. Figure 1 indicates the activated moieties are pyrophosphoric anhydrides, dihydropyridine, sugar aminals and acetals, allyl pyrophosphates, trivalent sulfonium ion, acyl phosphoric anhydrides, and acetyl thioesters.[8,12] Vastly different chemical functional groups have been recruited into biologically stable frameworks to be spent at times and places in cells determined by the availability and activity of cosubstrates and the requisite enzyme catalysts.

Table 1 indicates major metabolic pathways in (higher) eukaryotic cells with the one or more of these seven metabolites activated for group transfer driving key steps in those pathways. For numeric estimates of the substrates, including concentrations, K_{eq} values in different organisms, cellular compartments, etc., one can refer to the Bionumbers Web site (http://bionumbers.hms.harvard.edu/).[13]

1.2. Oxygen as a Thermodynamically Activated Inorganic Molecule

The definition of aerobic organisms is that they use molecular oxygen, O_2, as a metabolite. They do so carefully while reaping an enormous energy yield compared to organisms that run anaerobic respiratory chains and use alternate terminal acceptors with much less of a potential drop through which electrons are funneled.

Because of the energetics of how its molecular orbitals are filled, O_2 is a ground state triplet (essentially a stable diradical)[14] that reacts only sluggishly with spin-paired organic molecules, essentially all the molecules of primary metabolic pathways. Therefore, O_2 is kinetically stable and has accumulated to 20% of the earth's atmosphere. On the other hand, as noted earlier, it has an enormously favorable reduction potential, to undergo four-electron reduction to two molecules of water (Figure 2b). Without getting into the analytics of Nernst potential equations,[15] we noted above that the K_{eq} for passage of electrons from 2 NADH to O_2 to yield 2 H_2O is about 10^{38} in favor of electron flow as written.

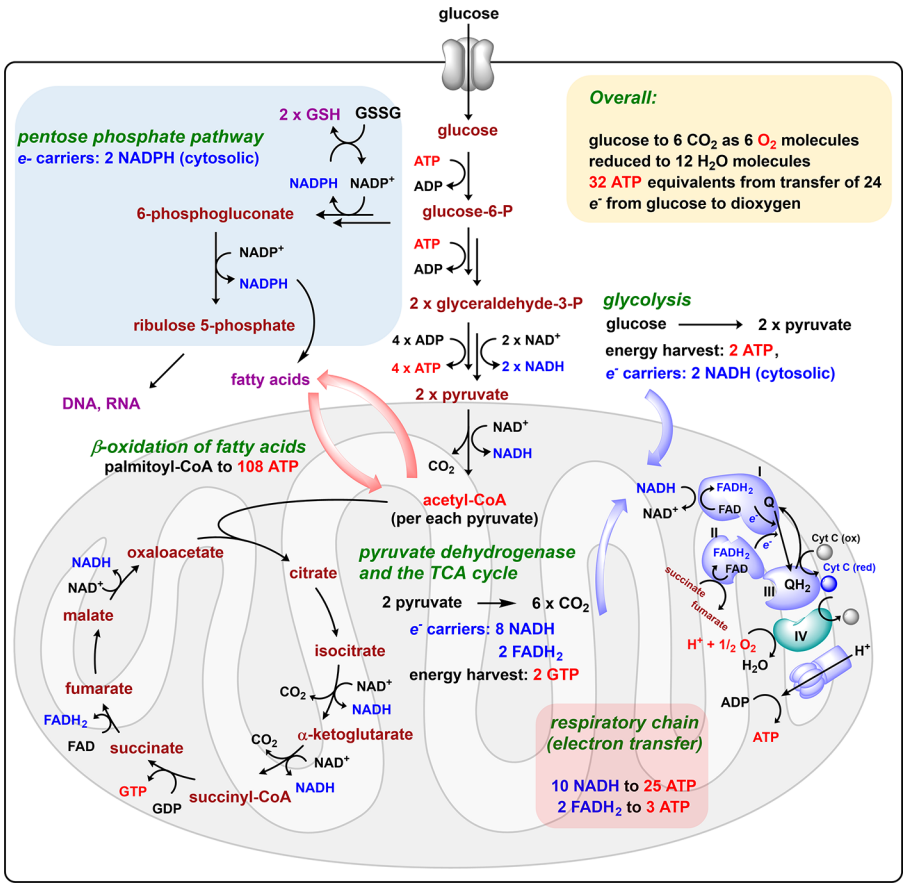

Figure 3. An overview of cellular glucose metabolism. The coupled pathways of glycolysis, TCA cycle, and electron transport down the membrane respiratory chain summarize removal of 24 electrons from each glucose molecule and ~30 ATP molecules accumulated for each four electron reduction of O_2. A second major flux of glucose carbons is via the pentose phosphate pathway, yielding 2 equiv of NADPH used for fatty acid biosynthesis and to make D-ribose-5-P for nucleic acid biosynthesis. The diagram also notes that complete oxidation of the predominant C_{16} fatty acyl-CoA, palmityl-CoA, to 8 molecules of acetyl-CoA that are run through the TCA cycle and respiratory chain yields ~108 ATPs, emphasizing that saturated fatty acyl chains are energy storage molecules.

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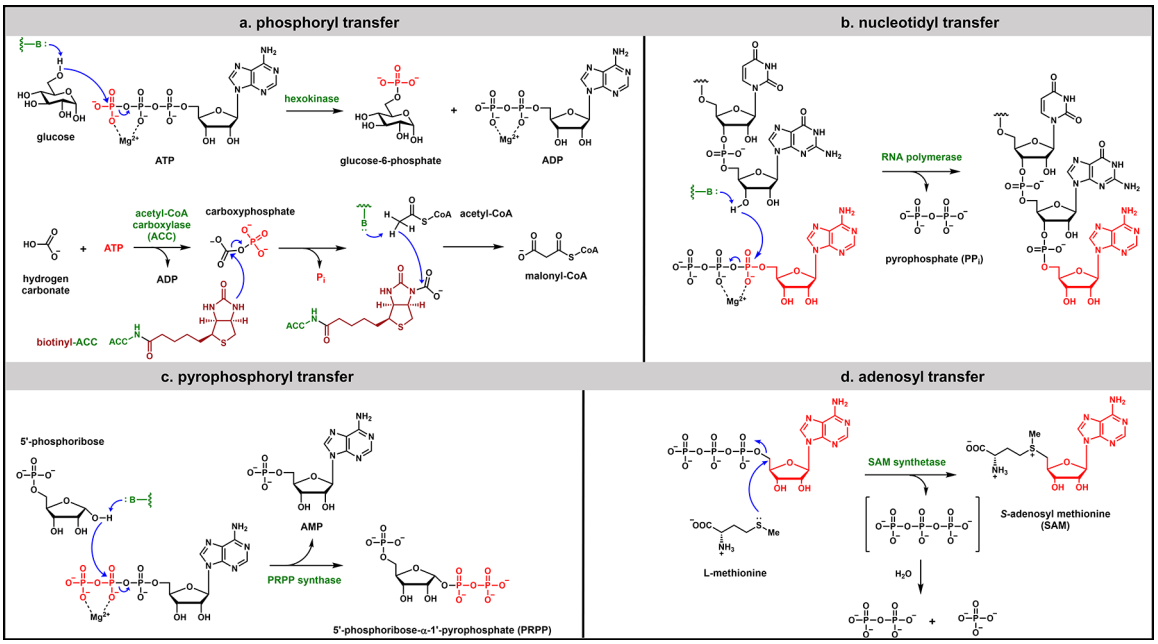

Figure 4. Mechanistic alternatives for ATP side chain cleavage to drive coupled equilibria: (a) phosphoryl transfer involves attack of cosubstrate nucleophile on electrophilic γ-phosphorus atom of Mg-ATP; (b) attack of cosubstrate nucleophile on α-P of Mg-ATP constitutes nucleotidyl (here adenylyl) transfer and is the common side chain cleavage mode in RNA, DNA, and protein biosynthesis; (c) pyrophosphoryl transfer involves cosubstrate attack on the β-P of Mg-ATP and is relatively rare, although the formation of PRPP is central to purine biosynthesis; and (d) the fourth mode of ATP side chain cleavage involves attack of cosubstrate nucleophile on C_5' of the ribose moiety of ATP and, while also a metabolically rare cleavage mode, is central to the formation of *S*-adenosyl methionine.

Thus, O_2 is kinetically stable but thermodynamically activated and joins the other seven molecules noted in the previous sections in this key attribute. One could argue that metabolism is all about selectively parsing/unleashing those seven thermodynamic potentials in the active site microenvironments of enzymes to direct flux to specific product metabolites. One clear distinction between the prior seven thermodynamically activated, kinetically stable metabolites is that they are all organic molecules. O_2 by contrast is an inorganic molecule. Since nearly all of the 20% of the earth's atmosphere that is O_2 has been biogenically derived via O_2-producing photosynthesis.

2. ATP

ATP is the key metabolite from both the glycolytic pathway in cell cytoplasm and from the combined action of the tricarboxylate cycle and respiratory chain function in mitochondria. The net production of ATP from the ten enzyme glycolytic pathway is two ATPs per glucose molecule broken down to two molecules of pyruvate (Figure 3). A net of 32 ATPs is realizable from passage of all 24 glucose-derived electrons down the respiratory chain to O_2 as terminal acceptor (Figure 3).[10,16] This 16-fold disparity indicates the tremendous thermodynamic advantage of utilizing the favorable thermodynamics of reduction of O_2 to H_2O (see section 4) and of taking all 24 electrons, as opposed to four electrons (at the end of glycolysis), out of glucose.

ATP approaches perfection as a cellular packet of molecular energy currency. It is kinetically stable under physiologic conditions, despite containing two pyrophosphoric anhydride linkages in its triphosphate side chain.[7,17] Anhydrides are uphill energetically from their hydrolyzed acid products. In the case of ATP, this is ∼−7.3 kcal/mol (−30.5 kJ/mol), corresponding to an equilibrium constant favoring hydrolysis of 10^5 (Figure 2a).[18] Thus, cleaving ATP to ADP and P_i or to AMP and PP_i (−45.6 kJ/mol) creates a large driving force to couple otherwise unfavorable processes. The kinetic basis of stability lies in the ionization of the phosphate side chains such that ATP is tetra-anionic at physiologic pH, and water and other adventitious electron-rich atoms cannot readily penetrate that phalanx of repulsive negative charges. Indeed, ATP is stable enough to be used by thermophilic microbes. In every enzymatic reaction, it is Mg-ATP that is the substrate, with divalent Mg^{2+} serving to shield two of the side chain negative charges in a given enzyme active site and enable directed nucleophilic attack and side chain cleavage.

ATP is spent in every anabolic pathway in cells for both low molecular weight and high molecular weight metabolites. ATP experiences two major modes of side chain triphosphate cleavage, reflecting two different families of cleaving enzymes, as it drives hundreds of millions of coupled reactions in every cell (Figure 1). The global concentration of ATP in a cell is estimated to range between 0.5 and 5 mM[19] but could differ in subcellular compartments from moment to moment and can be higher in metabolically active tissues such as muscle. At these concentrations, many, if not most of the myriad enzymes that utilize ATP (e.g., the proteasome for degradation of short-lived proteins) will be saturated with this equilibrium-driving cosubstrate. Kornberg has estimated that eukaryotic cells contain on average 10^9 molecules of ATP.[20]

2.1. Phosphoryl Transfers

One mode is phosphoryl transfer where a cosubstrate nucleophile, most commonly an oxygen atom attacks the γ-phosphorus, with net transfer of PO_3^{2-}. For example, this is the reaction catalyzed by hexokinase (Figure 4a), trapping glucose inside cells as glucose-6-P, with an equilibrium constant of around 10^3, and functionally irreversible. It is also the mode of

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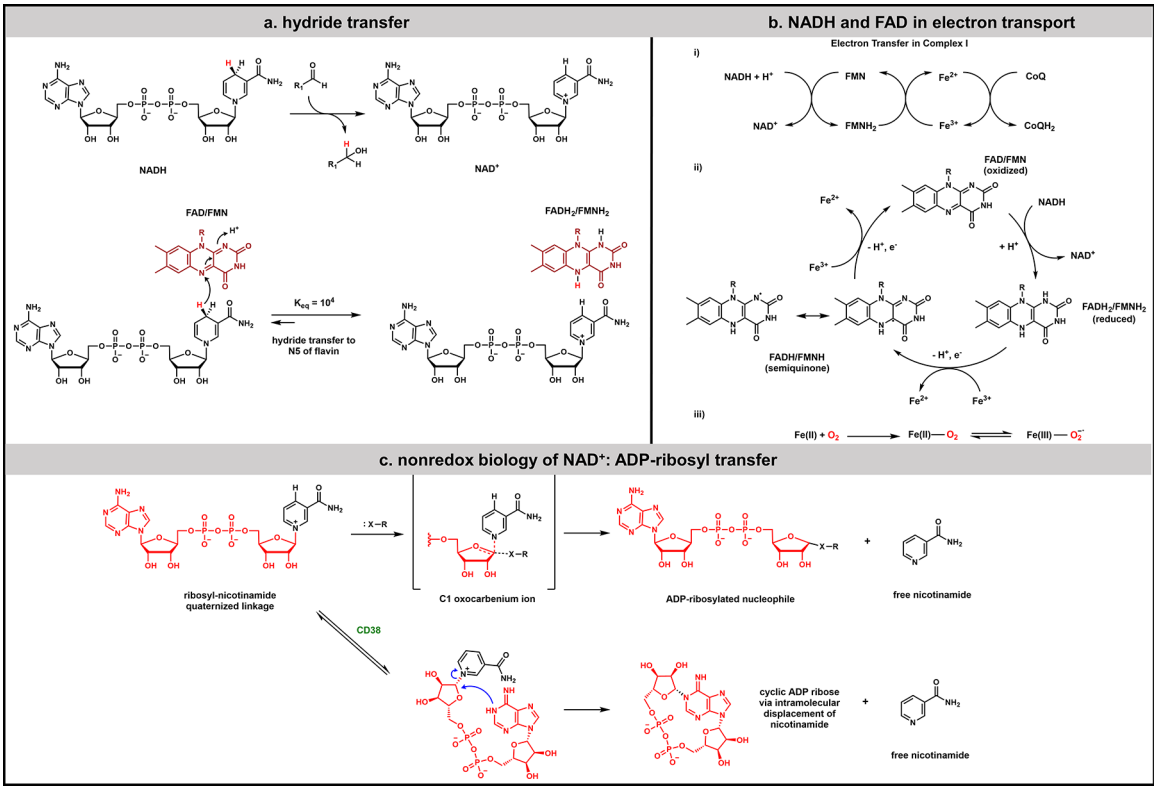

Figure 5. Two modes of reactivity of oxidized nicotinamide coenzymes: (a) the most common role of NAD(P)$^+$ is as a hydride acceptor during cosubstrate oxidation to form NAD(P)H. In the back direction NAD(P)H is a thermodynamically activated hydride donor for cosubstrate reductions. (b) FAD has a special place in NAD(P)H biochemistry as a favored acceptor of a hydride to give $FADH_2$. The dihydroflavin can subsequently be reoxidized either by a reverse of the two electron pathway or by two one electron steps. FAD (and FMN) thus functions as a 2 electron/1 electron step down redox transformer and interfaces between NAD(P)H and obligate one electron acceptors such as O_2 and Fe^{3+} proteins. (c) Nonredox biology of NAD$^+$ involves ADP-ribosyl transfer to nucleophiles. These can be side chains on proteins or growing ADP-ribosyl chains. Also shown is the comparable intramolecular displacement on NAD$^+$, creating cyclic ADP ribose.

action of phosphofructokinase in step 3 of glycolysis as fructose-6-P is converted to fructose-1,6-P_2 in preparation for aldolase cleavage.[17]

The gatekeeper enzyme in fatty acid biosynthesis, acetyl-CoA carboxylase,[21] uses ATP cleavage to activate bicarbonate, as carboxy phosphate, for the biotin-dependent C−C bond-forming step to yield malonyl-CoA (Figure 4a). In steroidogenesis, three ATPs are cleaved to ADP sequentially as mevalonate is processed to Δ^3-isopentenyl-PP, the building block to all isoprenoid downstream metabolites (see Figure 11).[22] Of the ten enzymatic steps required to build the purine ring building blocks for RNA and DNA, six of those enzymes cleave ATP at the γ-phosphoryl group. All ∼500 protein kinases in the human kinome also use this mechanism for post-translational modifications of myriad protein substrates in signal transduction manifolds with rate accelerations up to 10^{11} over uncatalyzed transfer.[23]

2.2. Nucleotidyl Transfers

The second mode of ATP cleavage, attack of a cosubstrate nucleophile at α-P, defined as nucleotidyl transfer, again breaks a phosphoric anhydride linkage and so drives coupled equilibria by a 10^8 increment (Figure 4b).[7,8] This is the key cleavage mode in four biosynthetic pathways to biological macromolecules: proteins, RNA, DNA, and oligo- and polysaccharides. All aminoacyl-tRNA synthetases generate aminoacyl-AMPs in the first step of their catalytic cycles before transfer of the now activated aminoacyl moiety to cognate tRNAs. Analogously, RNA polymerases transfer XMP moieties from XTP (X = A, G, C, and U) monomers during chain elongation by RNA polymerases. In strict analogy, DNA polymerases transfer dXMP fragments from dXTPs (X = A, G, C, and T).

The preference for XMP (nucleotidyl) transfer to cosubstrate nucleophiles over phosphoryl transfer in the thermodynamically uphill accumulation of proteins, and nucleic acids may be due to the presence of active pyrophosphatase enzymes in cells (Figure 4b).[24] The PP_i coproduct released in nucleotidyl transfers is hydrolyzed to 2 molecules of inorganic phosphate, providing another kick of 10^3 in the equilibrium constant in the forward, biopolymer-accumulating, direction. The activity of pyrophosphatases is enough to keep [PP_i] levels at micromolar levels or below, drawing off the equilibria in the front direction. The coupling of nucleotidyl transferases and pyrophosphatase tandem action makes NMP transfers the most effective NTP/ATP cleavage pattern for accumulating biopolymers. The flux of nucleoside triphosphates, via NMP transfer, to drive DNA replication (∼2 × 10^8 ATP to replicate the *E. coli* chromosome), RNA transcription (∼200/typical mRNA of 1000 nucleotides), and the many peptide bonds in each protein (∼1500 ATP per 30 kDa protein) may be the largest energy drain in proliferating cells.[13]

2.3. Pyrophosphoryl and Adenosyl Transfers

Attack of cosubstrate nucleophiles on β-P of ATP to fragment the α, β-phosphoric anhydride linkage and get the 10^8 boost in equilibrium can occur, although this regiochemistry is quite rare

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in metabolic pathways.[8] One example at the start of the purine biosynthetic pathway is the enzyme PRPP synthase[25] which converts 5-phosphoribose (from the pentose phosphate pathway noted below in discussion of NADPH formation) to 5-P-ribose-α-1-PP (PRPP) with release of AMP.[26] In the very next step, catalyzed by amidophosphoribosyltransferase, nascent NH_3, generated via glutaminase action, displaces the PP_i at C_1 of the ribose moiety and generates 5-P-ribose-β-1-amine, the starting point for elaboration of the imidazole ring of AMP (Figure 4c).

There are two well-described enzymatic reactions in which ATP is attacked at the 5′ carbon of the ribose moiety, for unusual adenosyl transfers, rather than any of the three electrophilic phosphorus side chain atoms. One is in the formation of *S*-adenosylmethionine (SAM) by SAM synthetase, discussed in section 7 below (Figure 4d). The other is in the biosynthesis of coenzyme B_{12}, carried out by bacteria, including those in human gut microbiomes to provide this essential vitamin to the host. Salmonella have three adenosyltransferases, operating under different conditions.[27,28] The reaction requires reduction of the cobalt in the corrin ring of the vitamin B_{12} cosubstrate from Co (II) to nucleophilic Co (I) as the species attacking C_5' of ATP. The nascent product from side chain cleavage is the inorganic triphosphate which can be cleaved by pyrophosphatases to draw the equilibria in the biosynthetic direction.

In sum, ATP, and the other three common nucleotide triphosphates (GTP, CTP, and UTP) are perfectly balanced between kinetic stability and thermodynamic activation. The phosphoric anhydrides (α-P-β-P, β-P-γ-P) shielded by the cloud of anionic charges, are the structural keys and could not have been replaced with neutral carbon-based anhydrides which are hydrolytically labile. Finally, the negative charge makes ATP and related purine and pyrimidine nucleotides unable to cross cell membranes and requires population of ATP pools in each cellular organelle, most notably in nuclei and cytoplasm as well as mitochondria, to drive organelle-specific pathway steps.

3. NADH AND NADPH

3.1. Molecular Basis of the Utility of NADH

The second-most cited molecule connoted as "energy rich" in cell metabolism is NADH. The oxidized form NAD^+ has recently been touted as an antiaging metabolite.[29,30] The assessment of energy released and stored in glycolysis includes the two NADH molecules formed during glyceraldehyde-3-P dehydrogenase catalysis, reflecting storage of four electrons removed from the starting glucose molecule up to that point (Figure 3). Then the conversion of two molecules of pyruvate to the 6 CO_2 final product molecules at the end of the TCA cycle yields another 8 NADH molecules within the mitochondria. The remaining four electrons that have been removed, ultimately from the pyruvate scaffolds, are stored as two Enz-$FADH_2$ equivalents. It is most productive to think of NADH as packets of cellular electron transfer currency, via hydride group transfer (Figure 1): the two electrons move with the hydrogen atom.

One can ask why is NADH a thermodynamically activated metabolite and how does it funnel electrons to O_2 to make all those ATP molecules? The answer to the first question lies in the chemical nature of the reduced form of the coenzyme(s) NADH and NADPH. Dozens of NAD^+-dependent reductases all catalyze hydride transfer from a cosubstrate undergoing oxidation to the 4-position of the positively charged nicotinamide ring, yielding the dihydronicotinamide. This is uphill energetically from the resonance-stabilized pyridinium ion of the oxidized nicotinamide (NAD^+) and is thermodynamically activated to revert back to the oxidized form, gaining aromaticity, and giving up the hydride ion (Figure 5A).

Given the two available oxidation states, NAD^+ and NADH, the predominant form in cells is the oxidized NAD^+, the thermodynamically more stable form, in ratios estimated from 10/1 to 700/1 for NAD^+/NADH,[31−33] with a total concentration estimated around 0.3−1.0 mM.[34] At 1 mM levels, there would be about 2×10^9 molecules of total NAD^+/NADH per human cell. The ratio in different organelles can clearly vary (e.g., in mitochondria carrying out TCA cycle flux where NADH will accumulate transiently). The concentration of O_2, normoxia or hypoxia, will also affect NADH concentrations in mitochondria, affecting respiratory chain electron flux.

NADH and NAD^+ levels oscillate with circadian rhythm, connecting circadian and metabolic cycles.[35,36] A variety of mechanisms could be involved in sensing and determining cause and effect. SIRT-1 and PARP-1, which both modify the covalent state of clock proteins (deacetylation and ADP ribosylation, respectively) could be contributors to circadian and metabolic coupling.[35−37]

The most common measure of the tendency of NAD(P)H to give up the hydride and associated two electrons is its reduction potential.[38] At physiologic pH and temperature, the reduction potential for the NADH/NAD^+ couple is −320 mV (Figure 2b). The corresponding potential for the vitamin B_2-based coenzyme forms FMN and FAD are in the range from −220 to −200 mV. Thus, the equilibrium for electron transfer of NADH to Enz-FAD is 10^4, consistent with the observation of many flavoenzymes reduced by NAD(P)H in cellular metabolism (Figure 5a). The corresponding reduction potential for O_2 to 2 H_2O is +820 mV. So, if there would be a route for transfer of electrons from NADH to O_2, the electrons would fall through a potential drop of 1.14 V which translates to an equilibrium constant, by Nernst equation analysis, of about 10^{38} (a factor of 10 in K_{eq} for every 30 mV of potential difference) (Figure 2b).

This is the basis for why aerobic metabolism has such a high energy yield. Each passage of two electrons down the mitochondrial membrane respiratory chain to O_2 leads to enough protons flowing back into the mitochondria through ATP synthase machinery to make ∼2.5 ATPs.[10] The corresponding flow of electrons from $FADH_2$ to O_2 yields ∼1.5 ATPs per electron pair (Figure 3). Passage of the 24 electrons from 10 NADH and 2 $FADH_2$ to O_2 yields 28 ATPs from the conversion of two pyruvates to 6 CO_2 molecules.

Additional facets of NAD^+/NADH reactivity and utilization affect the workings of cellular metabolism. First is the observation that NADH and NADPH are restricted to two electron transfer steps. Hydride transfer but not hydrogen atom transfer (H•) is observed.[8] The one electron pathway, generating a pyridinyl radical, is too high in energy (reduction potential in the −600 mV range) and is out of range of biological reagents[38] and does not demonstrably occur in metabolism. Because both the Fe^{3+} cytochromes in the mitochondrial respiratory chain and O_2 itself are obligate one electron acceptors, there is a mechanistic incompatibility with the thermodynamic propensity for electrons to flow from NADH to cytochromes to O_2.

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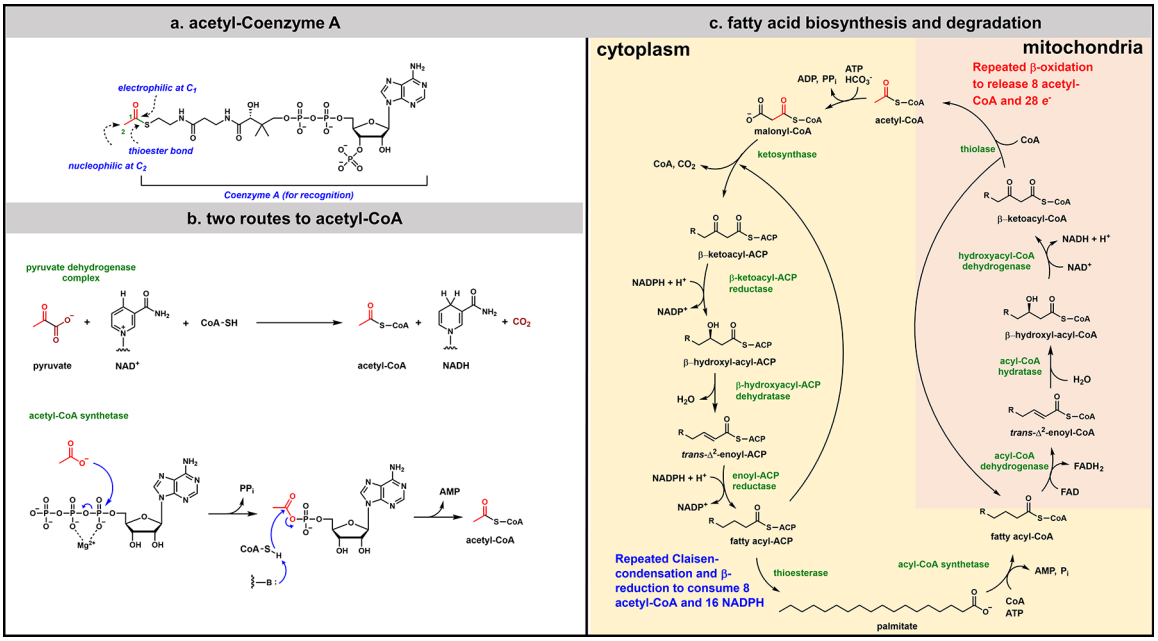

Figure 6. Overview of Acetyl-CoA: (a) structure and chemical features of acetyl-CoA; (b) two biosynthetic routes to acetyl-CoA involve (1) the oxidative decarboxylation of pyruvate to acetyl-CoA in the transition between glycolysis and TCA cycle catalyzed by the multisubunit pyruvate dehydrogenase; (2) the conversion of acetate back to acetyl-CoA by nucleotidyl transfer and acetyl-AMP intermediacy (free acetate in mammalian cells can arise by multiple routes including histone deacetylase action); (c) in times of energy excess, the utilization of acetyl-CoA carbons for fatty acid biosynthesis occurs in cell cytoplasm while the reverse steps occur during mitochondrial beta oxidation of fatty acids to generate acetyl-CoA molecules.

A key consequence of the restriction to two electron redox routes is that NAD(P)H is not readily autoxidized by O_2, despite the favorable 10^{38} equilibrium. Thus, NAD(P)H joins ATP as a chemical invention in nature where thermodynamic activation has been built in to a scaffold that is kinetically stable in cellulo. In turn, this kinetic stability makes NADH and NADPH ideal packets of diffusable two electron transfer currency, via thermodynamically favorable transfer of a hydride group, for hundreds of cellular reactions.

3.2. Nicotinamides and Flavins

The question arises of how electrons get from NADH to cytochromes and then to O_2. One or more two electron/one electron step down electron transfer molecules must intervene.[39] That molecule is the other common redox coenzyme in cellular metabolism: the coenzyme forms, FMN and FAD, of riboflavin (Figure 5b). The planar tricyclic ring system of flavins can accept one electron or two electrons at a time, to yield the semiquinone radical or the two electron reduced dihydroflavin. Because the unpaired electron can be delocalized and thereby stabilized through the tricyclic ring system, the energy is lowered, the radical is stabilized, and is kinetically accessible in cellular metabolism.

Thus, flavins are the intermediary metabolites between NAD(P)H and obligate one electron acceptors, including both Fe^{3+} heme and nonheme centers in acceptor proteins and also O_2 itself (in oxygenase catalysis). A canonical pattern of electron flow for passage of electrons to the respiratory chain and O_2 is schematized in Figure 3 and Figure 5b.[40]

A corollary to the one electron reactivity of flavins and the autoxidizability of $FMNH_2$ and $FADH_2$ forms (half times for reoxidation by dissolved oxygen (~0.24 mM) less than a second), is that, unlike NAD(P)H, they are not suited to function as freely diffusing electron transfer packets in cells, and they do not. FAD and FMN are tightly bound to respective apoproteins, and the $FMNH_2$ and $FADH_2$ states typically even more tightly bound, to avoid adventitious autoxidation and interruption of electron flow to specific partner acceptors.[39] For this reason, flavins, unlike the more air stable NAD(P)H and $NAD(P)^+$, do not serve as freely diffusing substrates in cells. Instead they function as bound coenzymes. They are chemically more versatile than the nicotinamides but physically more restricted.

As with ATP, the negatively charged phosphate groups in NAD^+ and the additional one in $NADP^+$ provide electrostatic barriers to passage of nicotinamides across organelle membranes, in particular between cytoplasm (site of glycolysis and NADH production from GAPDH action) and mitochondria. As noted below, in section 10, transporters for malate, along with malate dehydrogenases in both cytoplasm and mitochondria, are one way of moving reducing equivalents (hydride ion transfers between the malate and oxaloacetate diacid substrates) between organelles (see Figure 17).

3.3. Differential Role for NADPH vs NADH

The extra phosphoryl group at the 2′-OH of the ribose moiety of the 2′,5′-ADP portion of NADP(H) (Figure 1) allows differential recognition from NAD(H) by cellular dehydrogenases and so allows specialization of function. In particular NADPH, generated in the cytoplasm of cells via the pentose phosphate pathway of glucose flux (Figure 3),[41] serves as the hydride donor in the enoyl reductase step of each cycle of fatty acid biosynthesis, reducing the CH=CH group to the bis-methylene CH_2-CH_2 group representing the fully reduced acyl chain (see Figure 6c). NADPH is also the reductant, utilized by glutathione reductases, for converting the oxidized, disulfide form of the tripeptide glutathione back to the thiol form in reduced glutathione (GSH) (Figure 3).[42] In turn, GSH is used

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by a family of glutathione *S*-transferases and peroxidases to detoxify electrophilic xenobiotic molecules and also toxic partially reduced oxygen molecules (superoxide, hydrogen peroxide) in cells.

The concentration of NADPH in cells is thought to be around 10% the total concentration of NAD^+/NADH, which would put it around 100 μM ($\sim 2 \times 10^8$ molecules/human cell). In contrast to NAD^+/NADH where the oxidized form is predominant, the NADPH/$NADP^+$ ratio can be as high as 20 to 200/1.[31−33] This is consistent with significant flux through the pentose phosphate cycle to generate the reduced form of this coenzyme and its utilization to drive the kinds of metabolic reactions noted in the preceding paragraph. Additional sources of NADPH can be malic enzyme action,[43,44] mitochondrial IDH2,[45] NAD^+ kinases that convert NAD^+ to $NADP^+$ frameworks,[46] and transhydrogenase equilibration of hydride transfer between NADH and $NADP^+$.[47]

3.4. Nonredox Biology of NAD^+: ADP-Ribosyl Transfers

In addition to the thermodynamic activation of the NADH oxidation state to deliver a hydride ion to cosubstrate carbonyl groups as it rearomatizes to NAD^+, the NAD^+ oxidation state is also thermodynamically activated for a different type of nonredox group transfer. That is ADP-ribosyl transfer.[12,48] As shown in Figure 5c, the cosubstrate nucleophiles could be an internal nucleophile or water in NAD^+ cyclohydrolases or, in a chemically unlikely step, acyl groups on protein deacylases in the family of enzymes known as sirtuins.[49,50] This ADP-ribosyl transfer capacity is available in NAD^+ but not NADH because the cationic pyridinium ring is a low-energy leaving group while the dihydroaromatic ring in NADH is not. Because NAD^+, not NADH, is the predominant redox state in cells, NAD^+ is readily available for ADP-ribosyl transfers.

The most celebrated of the sirtuins remove acetyl groups from lysine residues in the *N*-terminal tails of histones, cleaving an NAD^+ molecule in each catalytic cycle as the acetyl group is transiently stored on the ADP-ribosyl moiety of NAD^+ during net hydrolysis.[51,52] These NAD^+-dependent histone deacetylases, more generally acting as protein deacetylases, are involved in transcriptional control and have been interpreted as directly connecting NAD^+ levels to patterns of gene expression.[53,54] Other sirtuins have been demonstrated to remove negatively charged malonyl groups, succinyl groups, and longer chain acyl groups from proteins in different cellular compartments.[55,56]

A third set of ADP ribosyl transfers occurs in the nucleus under the catalytic agency of a family of poly-ADP-ribosyl polymerases (PARPs). They are activated during DNA damage and stress situations and the PARPs build chains of anionic ADP-ribose polymers (PARs) tethered to specific proteins.[57] PARP isoenzymes have become targets of clinically effective inhibitors in cancer therapies.[58] PARP-1, the most active of 17 isozymes (half of which are PARPs, half monoADP ribosyl transferases), can make branched PAR chains that range from a few residues long up to ~240 ADPR residues.[59] PAR chains built on four kinds of protein side chains (glu ~ asp ~ lys ~ arg) can be cleaved to yield free PAR molecules by action of PAR glycohydrolases that generate ADP ribose monomers and the unmodified protein products.[60,61] The dynamics of PAR chain build up and breakdown can use up substantial fractions of cytoplasmic NAD^+ pools in cells; in turn, insufficient cytoplasmic NAD^+ can lead to cessation of glyceraldehyde-3-P dehydrogenase-mediated carbon flux and blockade of glycolysis.[62] The flux of cellular NAD^+ down these ADP ribosyl transfer pathways in higher eukaryotic cells can be substantial,[5] suggestive of their metabolic importance.

An intramolecular variant of ADP ribosyl transfer occurs in the enzymatic formation of cyclic ADP ribose by the protein CD38,[63] a second messenger for mobilization of intracellular calcium stores, where N_3 of the adenine ring of NAD^+ is the kinetically competent nucleophile as free nicotinamide is released (Figure 5c).

One last comment about NAD^+/NADH involves the anticipated turnover of this metabolite in cells. Given that ~2.5 ATPs are formed for each pair of electrons sent by an NADH molecule down the respiratory chain and that ~80 kg of ATP (MW = 554 Da) are turned over in a day per adult male human,[20] about 30 kg of NADH (MW = 664 Da) must be generated and funneled to O_2 every day. A substantial fraction of the electron equivalents in NADH in times of energy surfeit must exit the mitochondria by the high capacity malate shuttle noted above and in section 10 to drive cytoplasmic electron transfer metabolism (e.g., lactate dehydrogenase action in aerobic glycolysis noted in a subsequent section).

4. ACETYL-COA

4.1. Molecular Basis of the Utility of Acetyl-CoA

The third of the "big three" molecules to arise from the central catabolic pathway of cytoplasmic glycolysis and then mitochondrial oxidative decarboxylation of pyruvate is acetyl-CoA.[6] This is the vehicle by which the remaining two carbons from pyruvate get converted to CO_2 in the tricarboxylate cycle. Acetyl-CoA is also generated during fatty acid catabolism as will be noted below.

At least as interesting in terms of roles in metabolism, acetyl-CoA powers two central anabolic pathways: fatty acid biosynthesis and isoprenoid biogenesis, including all the steroid scaffolds.[6,64] The acetyl group is also used in a plethora of post-translational modifications of proteins, among them the enzymatic acetylation of histone tails in chromatin, with attendant control of transcriptional activation of genes.[65]

This central metabolite has two parts: (a) the coenzyme A moiety that serves both as recognition module for many enzymes and crucially provides the terminal nucleophilic thiol group and (b) the acetyl moiety that is attached to the thiol in acyl thioester linkage (Figure 6a). The acyl thioester group is the chemical basis for the utility of acetyl-CoA as a thermodynamically activated acetylating agent of cosubstrates. The K_{eq} for hydrolysis to acetate and CoASH is $\sim 10^6$, the same favorable equilibrium as for ATP hydrolysis, a measure of the driving force that acetyl transfer provides for transfer to water oxygens (net hydrolysis) or to cosubstrate nucleophilic oxygen, nitrogen, and sulfur atoms.

Acetyl-CoA can also be generated enzymatically de novo from acetate, ATP, and free coenzyme A in a two step process (Figure 6b).[66−68] This involves attack of the acetate carboxylate group on α-P of Mg-ATP to yield acetyl-AMP. This mixed acyl phosphoric anhydride is thermodynamically activated but kinetically unstable in cellular milieus.[8] It can be captured by the thiol of CoASH to yield acetyl-CoA and AMP via a committed enzyme, with a K_{eq} for this step close to 300 (Figure 2c). The accumulation of the activated acyl thioester has been driven by the net hydrolysis of ATP to AMP and PP_i but now the acyl thioester is much more stable than the acyl-AMP (hours for acetyl-CoA vs seconds for acetyl-AMP at physiologic pH and temperature) and is usable as a diffusible source of

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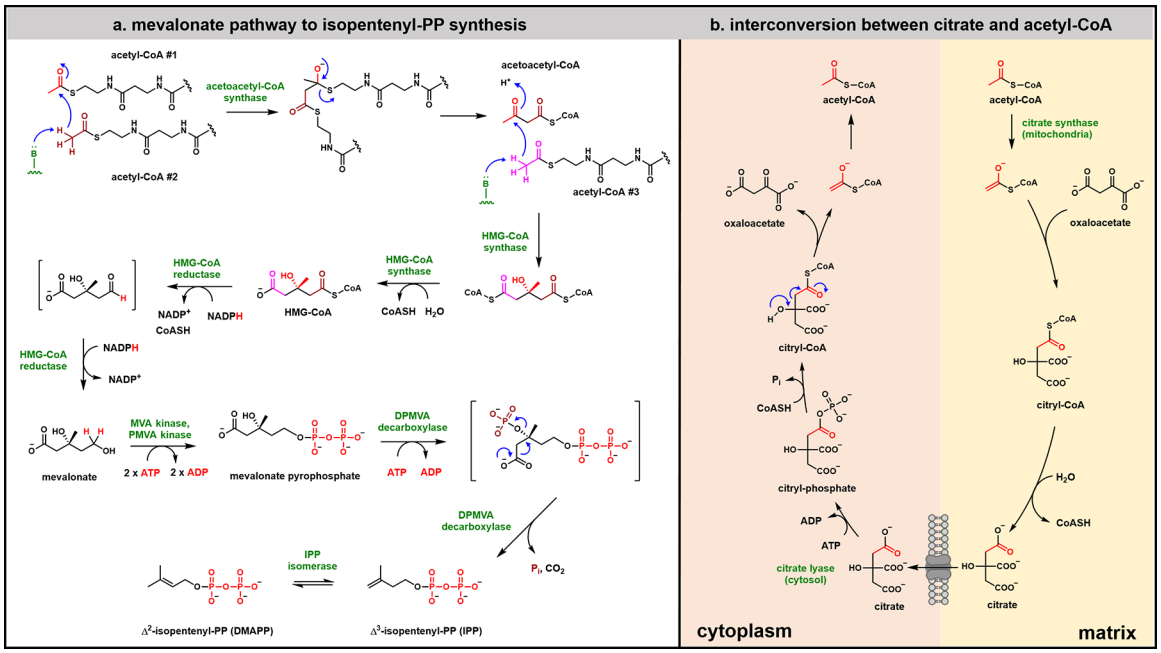

Figure 7. Acetyl-CoA metabolism part two: (a) conversion of three molecules of acetyl-CoA to the Δ^2 and Δ^3 isomers of isopentenyl diphosphate (IPP) by the enzymes of the mevalonate pathway. Note the utilization of three molecules of ATP and two of NADPH as HMG-CoA is converted to Δ^2-IPP; (b) acetyl-CoA and citrate can be interconverted by distinct enzymes in both cytoplasm and mitochondria and citrate can pass between the two compartments. Citryl-CoA is an intermediate common to both arms.

acetyl group transfers. We note the importance of this path for acetate to acetyl-CoA in tumor metabolism in a subsequent section.[69]

While acetyl-CoA is the prototypic, ubiquitously utilized acyl-CoA in many branches of primary metabolism, the combined kinetic stability/thermodynamic activation as an acyl transfer agent applies to other acyl-CoA metabolites as well and underlies their functions. Among them are malonyl-CoA (first committed step in fatty acid biogenesis-noted below), succinyl-CoA in the tricarboxylate cycle, and long chain fatty acyl-CoAs such as myristoyl (C_{14})- and palmitoyl (C_{16})-CoAs used in beta oxidation to release acetyl-CoA units in fatty acid catabolism as well as in protein post-translational protein acylations.

The acyl thioester strategy is also employed to generate intermediates in some key enzymes of primary metabolism. One example is in the NADH-forming step during glyceraldehyde-3-P dehydrogenase (GAPDH) action when a 3-P-glyceryl-*S*-Cys enzyme thioester is generated during NADH formation. A second set of widely distributed examples are the γ-glutamyl-*S*-enzyme thioester intermediates generated during glutaminase actions:[70] nascent NH_3 is released to be used as nucleophilic amination cosubstrate in many contexts of nucleotide and amino acid metabolism.[71]

There has been speculation that earliest organisms may have evolved without phosphorus, at low abundance in the earth's crust, and that acyl thioester chemistry may have been seen more central for metabolic acylation and C−C bond formations (see next section 4.2)[72] than in contemporary organisms. In such scenarios, it is not clear when and how the switch to phosphate-containing metabolites eventually would have become dominant in contemporary and retrieved fossil organisms.

4.2. Acetyl-CoA Doubly Activated in Anabolic Fatty Acid and Steroid Biogenesis

The chemical logic and protein machinery of fatty acid biosynthesis and steroidogenesis reveal that acetyl thioesters are actually doubly reactive metabolites, electrophilic at C_1 of the acetyl group and nucleophilic at C_2 of the acetyl group (Figure 6a).[9] Both features come into play in each pathway.

Fatty acid biosynthesis involves iterative thioclaisen condensation chemistry for C−C bond formation in each chain elongation step. Acetyl-CoA is first carboxylated to 2*S*-malonyl-CoA by the biotin-dependent multisubunit acetyl-CoA carboxylase, the gatekeeper enzyme for carbon flux into fatty acids for energy storage (Figure 6c). The malonyl-CoA can undergo transfer to the terminal thiol of acyl carrier protein domains in fatty acid synthase and undergo irreversible decarboxylation to the C_2 acetyl-CoA carbanion, stabilized by delocalization of the negative charge into the thioester carbonyl. This thioester enolate/carbanion equivalent is the attacking nucleophile on the elongating upstream acyl thioester chain, yielding a two-carbon elongated β-keto acyl thioester enzyme.

In every catalytic cycle of fatty acid synthase, this nascent β-keto acyl group undergoes four electron reduction. Both pairs of electrons come in from NADPH, the first reducing the β-keto to β-hydroxyl and the second transferring a hydride and a proton to reduce the enoyl-S-ACP. The net effect of the condensation and four electron reduction is chain elongation by CH_2-CH_2 unit. Two points to note: the thioclaisen condensation followed by four electron reduction occurs 7 or 8 times before release of the C_{16} and C_{18} fatty acids. In the case of the C_{16} palmitate, 28 electrons have been dumped in from NADPH (14 equiv), emphasizing that fatty acids are molecules with stored electrons/reducing equivalents that can be called on when cells need energy reserves mobilized. The second point is that the source of all the electrons are the reduced nicotinamide coenzyme.

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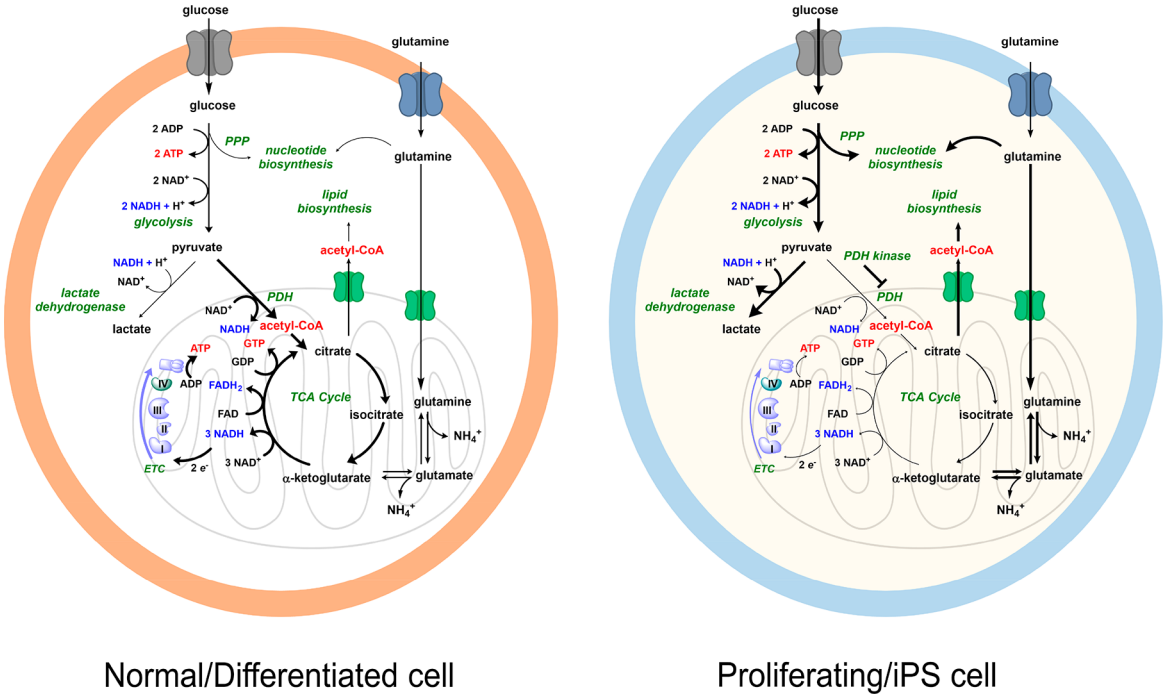

Figure 8. Comparison of metabolic fluxes from glucose and glutamine in normal/differentiating cells which are depicted with a robust TCA cycle and electron transport system vs proliferating IPS cells. Thick arrows indicate a greater flux through steps compared to thin arrows. The right-hand panel indicates less energetic flux through the TCA cycle and increased aerobic glycolysis. The ability to "dump" excess electrons and regenerate NAD^+ for glycolysis to continue depends substantially on high levels of lactate dehydrogenase, allowing reduction of pyruvate to lactate which can be excreted.

What goes up can come down in terms of fatty acid metabolism. In mobilization of the stored energy, the first step is transfer of the acyl chain from triglyceride to the thiol of coenzyme A by acyl transferases, acting as acyl-CoA synthetases. Enzymatic breakdown of long chain acyl-CoAs to acetyl-CoA units to send to the TCA cycle for catabolic energy production of ATP, the underlying chemical logic is the reverse of fatty acid synthase with the following distinctions (Figure 6c). Fatty acid β-oxidation typically occurs in mitochondria (although long chain fatty acids can be processed in peroxisomes) where the acetyl-CoA products can be funneled directly into the TCA cycle. The thioclaisen cleavages are carried out on soluble acyl-CoAs rather than acyl carrier proteins, allowing differentiation of the anabolic and catabolic arms of fatty acid metabolism. In each cycle where a CH_2CH_2 of the fatty acyl chain is converted to the CH_2CO-moiety that undergoes thiolytic cleavage and release as acetyl-CoA, the four electrons that are removed end up in NADH (not NADPH) and in $FADH_2$-enzyme molecules rather than two molecules of NADPH.

Reoxidation of NADH and $FADH_2$ by passage of the four electrons out to the respiratory chain complex I (NADH) and complex II ($FADH_2$) would yield 4 ATP molecules along with each acetyl-CoA released. Eight cycles of β-oxidation for complete degradation of palmityl-CoA would thus yield 32 ATP equivalents and eight acetyl-CoA product molecules. If all eight acetyl-CoAs were then processed to two CO_2 molecules via TCA action that would eventually yield 9 ATP and one GTP for each of eight turns of the cycle for another 80 ATPs (actually 72 ATPs and 8 GTPs). The total number of electrons removed from palmitoyl-CoA and sent down to oxygen are 96 electrons removed as 24 molecules of O_2 get reduced to 48 molecules of water. These numbers emphasize the energy storage functions of long chain fatty acids in cellular metabolism.

As noted in the paragraphs above, the relevant nicotinamide donor recognized by both the keto acyl-ACP and enoyl-ACP reductases in fatty acid synthesis is NADPH not NADH. NADPH is generated largely through cytoplasmic flux of glucose-6-P into the pentose phosphate pathway (noted below) (Figure 3). The first step in the pathway is $NADP^+$-mediated oxidation of C_1 of glucose-6-P from aldehyde to carboxylate oxidation state as NADPH is formed. Subsequent enzymes in this pathway equilibrate sugar scaffolds through actions of transketolase and transaldolase,[73] generating *inter alia* D-ribose-5-P which is a crucial building block for both purine and pyrimidine nucleotide monomers on the way to RNA and DNA biosynthesis. As noted above, NADPH is also the key reductant for oxidized glutathione by the enzyme glutathione reductase which keeps levels of the glutathione-SH form at high cellular concentrations to act as a protective scavenging metabolite.

The double-headed, orthogonal reactivity of the acetyl moiety of acetyl-CoA is further emphasized in the first two reactions of isoprenoid biosynthetic pathways. Thiolase catalyzes condensation of one acetyl-CoA as the C_2 carbanion with a second acetyl-CoA molecule offering up C_1 as the electrophile to yield the anticipated acetoacetyl-CoA: the same kind of C−C bond formation seen in fatty acid synthase (Figure 7a). The next enzyme takes a third molecule of acetyl-CoA and condenses it with acetoacetyl-CoA to form a branched chain six-carbon hydroxymethylglutaryl-CoA (HMG-CoA). In this reaction, the acetyl-CoA substrate is again used as a C_2 carbanion equivalent, attacking the C_3 ketone of acetoacetyl-CoA, building a new C−C bond in the branched framework.

To emphasize how NADPH and ATP, the other two central metabolites noted above, then mediate onward flux, HMG-CoA

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is reduced by two equivalents of NADPH to the corresponding alcohol mevalonate. Now ATP is used three consecutive times, all in phosphoryl transfers generating the mevalonate-PP metabolite at the primary alcohol before the third ATP phosphorylates the tertiary alcohol.[22] That last phosphorylation sets up a facile decarboxylation and loss of P_i to yield Δ^3-isopentenyl-PP (Figure 7a) (see section 6 below).

Acetyl-CoA undergoes the same kind of C−C bond formation in the first step of the TCA cycle, carried out by citrate synthase: the C_2 carbanion/thioester enolate is generated at the enzyme's active site and added into the C_2 ketone of cosubstrate oxaloacetate (Figure 7b). The nascent citryl-CoA is hydrolyzed to citrate, making the condensation and C−C bond formation irreversible. ($K_{eq} = 10^5$ for citryl-CoA hydrolysis). The TCA cycle then proceeds from this tricarboxylate to undergo a set of dehydration and rehydration to isocitrate and then two oxidative decarboxylations on the way to succinyl-CoA, half way through the cycle.

In these reactions involving acetyl-CoA and other acyl-CoA molecules, nature chose thioesters over the more available oxoesters because of the difference in orbital overlap of the carbonyl with adjacent sulfur versus oxygen atom. The corresponding acetyl-oxoesters would be 100 to 1000-fold less effective in activation of both the C_1 and C_2 of the acetyl moiety.[4]

4.3. Aerobic and Microaerophilic Glycolysis and Multiple Routes to Acetyl-CoA Pools

There are several examples of cells, including transformed cells,[74] T cells undergoing activation,[75] and pluripotent stem cells,[76,77] that practice aerobic glycolysis because the TCA cycle is not fully active for purposes of ATP synthesis. Thus, they do not oxidize glucose completely, and electrons do not get put into the mitochondrial respiratory chain effectively (Figure 8). In that state, the buildup of NADH could stop glycolysis by running out of cytoplasmic NAD^+. The safety valve in aerobic glycolysis is to reduce the pyruvate to lactate, dumping the electrons from NADH as L-lactate which is excretable. Each regenerated NAD^+ can now permit another glucose molecule to go through the ten enzyme glycolytic pathway to pyruvate. In this state, the activity of the NAD^+-dependent L-lactate dehydrogenase is a crucial catalyst, and its expression levels can be elevated.[74]

Canonically, acetyl-CoA is generated largely from the pyruvate dehydrogenase reaction in mitochondria when glucose is the predominant fuel. In times of energy deficits, acetyl-CoA units are obtained by fatty acid oxidation noted earlier. Depending on the energy needs of a given cell and the location of distinct subcellular pools of acetyl-CoA, distinct fates for this acetyl thioester substrate are possible. When the two acetyl carbons feed into the first step of the tricarboxylate cycle, eight electron oxidation ensues, all the way to two CO_2 molecules. Three NADH and one $FADH_2$-enzyme are the intermediate loci for capture and storage of the four pairs of electrons that have been removed. They can, between them, give rise to 9 ATP if all eight electrons get funneled down the respiratory chain rather than being used for other forms of cellular metabolic work. Alternatively, if the cell has enough ATP and NADH for all its needs, the acetyl-CoA can be used to build fatty acids and isoprenoid scaffolds in the cytoplasm as noted.

Recent studies have also indicated that T cell activation involves metabolic reprogramming to rely more on aerobic glycolysis, with overproduction of the L-lactate dehydrogenase *A* form tetramer as the key enzyme for regeneration of NAD^+ as noted above while pyruvate is reduced to lactate.[75] Less reliance on the TCA cycle for energy allows more release of citrate from the mitochondria and availability to the cytoplasmic ATP-dependent citrate lyase (Figure 7b). This enzyme generates the cytoplasmic acetyl-CoA that is utilized for fatty acid biogenesis as well as histone acetylations, subsequent gene activation, and transcription to drive the generation of the proteomic cellular profile (e.g., cytokines) and altered small molecule metabolites that comprise the activated state of the T cell subset.[75]

A fourth route to acetyl-CoA (to go along with pyruvate dehydrogenase, fatty acyl-CoA thiolase, and ATP citrate lyase) is via the action of the ATP-dependent acetate-activating acetyl-CoA synthetase enzymes found in the cell cytoplasm and nucleus and mitochondria (Figure 6b). This can be a consequential flux in some tumor cells that are either in hypoxic microenvironments and/or are practicing the aerobic glycolysis pattern noted above.[69,78] The free acetate required as substrate may arise from enzymatic hydrolysis of acetylated histones (halftime of acetylation estimated at 2−3 min[69,79] and other acetylated proteins and small metabolites).

4.4. Differentiation of Stem Cells: Switch from Glycolysis vs Respiration

Given the energy yield in ATP equivalents from (a) glycolysis versus (b) the combination of glycolysis, the tricarboxylate cycle and electron transport down the respiratory chain to molecular oxygen favors path (b) by 32/2 = 16-fold, one might anticipate all higher eukaryotic cells would run the latter program whenever possible. However, paradoxically, a hallmark of many cancer cells as noted above is a switch to glycolysis over glycolysis plus the TCA cycle, even in the presence of ample oxygen, the famous "Warburg effect".[80] More recently, it has become clear that pluripotent stem cells also are tilted toward glycolytic metabolism.[76,81] As they move down differentiation pathways, they reprogram metabolism and make more use of the TCA cycle and the respiratory chain. Less of the cellular energy flux comes from glycolysis (Figure 8). The reverse is true as iPS (induced pluripotent stem) cells are dedifferentiated back toward more primitive lineages: they begin to rely more on aerobic glycolysis than aerobic respiration. In both directions, the reprogramming of metabolism can be directed by selective gene transcriptional programs.

Although pluripotent stem cells are typically smaller, it is not obvious they have fewer mitochondria per cell volume than larger, differentiated cells. Those mitochondria may be less functional (e.g., immature in terms of enzyme or substructure complements) and may be more uncoupled with respect to electron flow to oxygen than fully mature mitochondria in differentiated cells, due to higher expressions of uncoupling proteins.[76,82] Pyruvate dehydrogenase is phosphorylated by PDH knase and is less active so less pyruvate may enter the mitochondria.[76] Instead, it is rerouted to lactate as high levels of tetrameric LDH A isoform are expressed. The NADH generated during glycolytic action of GAPDH is converted back to NAD^+ as pyruvate is rerouted reductively to excretable L-lactate (Figure 8). Thus, continual regeneration of NAD^+ allows for flux of glucose to pyruvate (and then lactate). Although only a net four electrons are taken out of glucose during its net conversion to two molecules of lactate, a high

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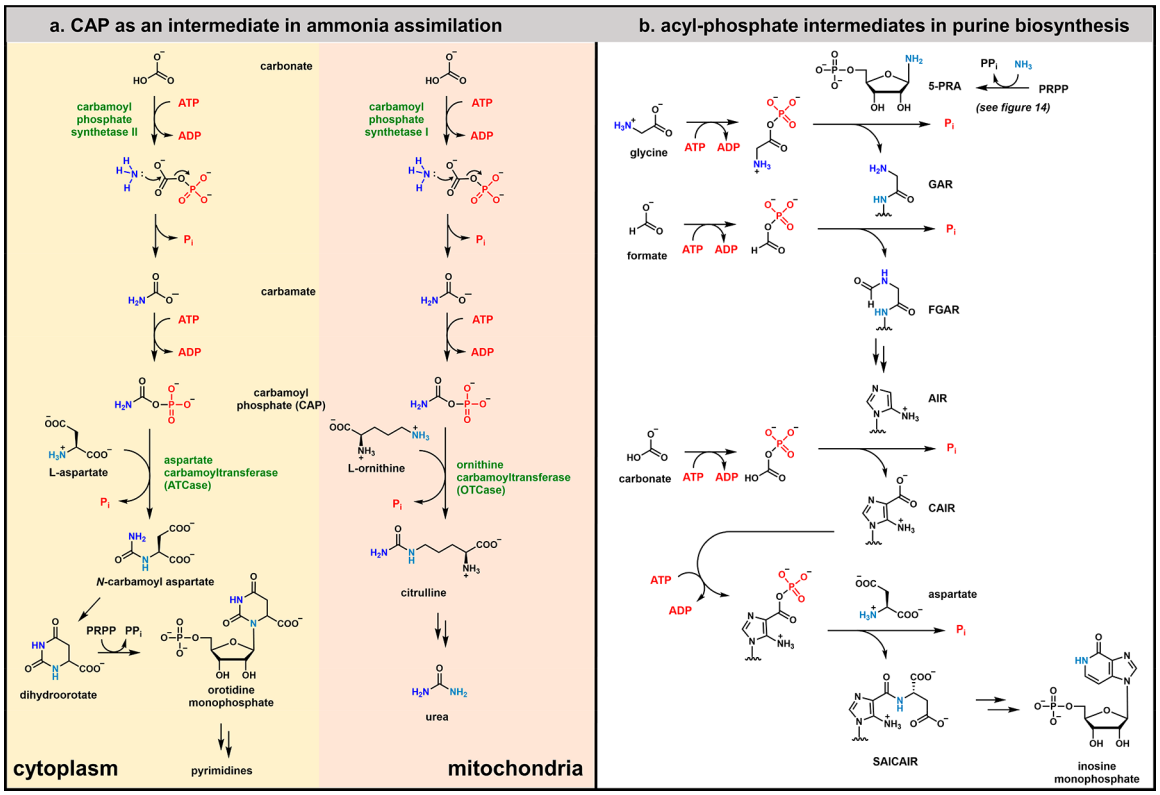

Figure 9. Carbamoyl phosphate and other acyl phosphates in purine biosynthesis: (a) carbamoyl-P is generated from carbonate, ammonia, and two molecules of ATP with carbonyl-P as an intermediate. Carbamoyl-P is central to mammalian nitrogen metabolism as a carbamoyl donor in anabolic metabolism, cytoplasmic pyrimidine biosynthesis, and in catabolic metabolism, mitochondrial urea synthesis. (b) The utility of acyl phosphates as kinetically stable, thermodynamically activated acyl transfer metabolites is emphasized in purine biosynthesis where four enzymes make glycyl-P, formyl-P, carbonyl-P, and CAIR-P as bound acyl donor species.

glycolytic flux appears to suffice to power stem cells. The TCA cycle runs at enough of a level to yield scaffolds for nitrogen-containing amino acids[83] and sufficient citrate, which presumably accumulates and can be transported to the cytoplasm as a source of acetyl-CoA.

A presumably higher reliance on mitochondria for energy/ATP in differentiating normal cells compared to highly proliferating tumor cells generates the metabolic reprogramming that allows higher numbers of functionally and structurally mature mitochondria to make more NADH (TCA cycle) and thereby more ATP (electron transport chain).

In stem cells that sit normally in hypoxic niches (e.g., hematopoietic stem cells in bone marrow microenvironments, mesenchymal stem cells, and neuronal stem cells), the hypoxic response allows nonhydroxylated HIF1α (see next section) to be long-lived as a key transcription factor to turn on a suite of genes. Among them is the above-noted pyruvate dehydrogenase kinase[76,77] that sends pyruvate dehydrogenase into an inactive state (Figure 8). Among the net effects is shutting down pyruvate entry into mitochondria (by mass action) and turning off acetyl-CoA production therein. In one sense, the switch back and forth, between glycolysis predominating or the full glycolysis/TCA cycle/mitochondrial respiratory chain predominating, is how cells can fare on different diets of NADH, ATP, and acetyl-CoA levels (both in the presence of glutamine as nitrogen source). Cancer or stem cells under hypoxic conditions or that exhibit anaerobic glycolysis may have induced glucose uptake, mediated by HIF1-mediated upregulation of the GLUT1 transporter to help compensate for the reduced yield of ATP from each glucose metabolized.[84]

5. CARBAMOYL PHOSPHATE, RELATED ACYL PHOSPHATES, AND GLUTAMINE

Carbamoyl phosphate, the mixed anhydride of carbamic acid and phosphoric acid, can be viewed as one of the anchor metabolites of nitrogen metabolism, acting both anabolically in pyrimidine biogenesis and catabolically in urea synthesis as a carbamoyl donor. The -NH_2 moiety of carbamoyl-P derives from nascent ammonia which is kept at low concentrations in cellulo because it is adventitiously reactive as a nucleophilic amine. The great bulk of cellular ammonia derives from the carboxamido group of glutamine by action of glutaminase enzymatic domains that release free NH_3 only in the glutaminase active site microenvironments. The nascent ammonia is efficiently captured by cosubstrates before unwanted release into the general cellular milieu.[85,86]

Although glutamine is the kinetically stable carrier and donor of ammonia for most anabolic steps in nitrogen assimilation, it is not typically thought of as thermodynamically activated analogous to the title molecules in this review. However, it, along with glutamate (available from glutaminase action) and aspartate, is also an anchor molecule for biosynthetic nitrogen metabolism. Further, the biosynthetic enzyme that traps ammonia and stores it as glutamine, glutamine synthetase, spends an ATP to sequester NH_3, a measure that the Gln γ-carboxamide is uphill from the Glu γ-carboxylate.

5.1. Carbamoyl- and Related Acyl-Ps

Carbamoyl phosphate (CAP) plays a key role in two pathways in mammalian nitrogen metabolism: (1) in the urea cycle for safe excretion of nitrogen atoms in nonnucleophilic form and

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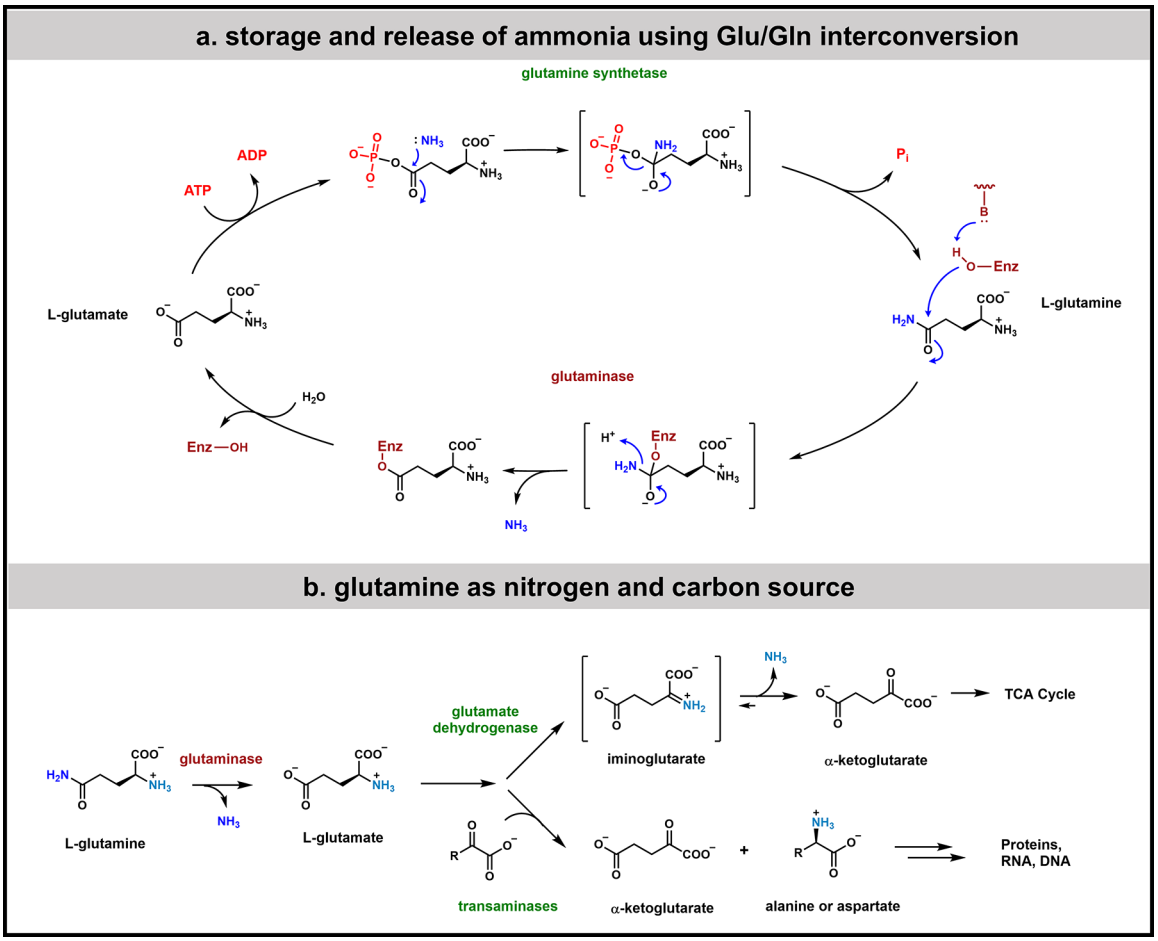

Figure 10. Glutamine as a nitrogen source: (a) glutamine and glutamate as key amino acids in balancing the storage and release of ammonia. Glutamine and glutamate are interconverted by the enzymes glutamine synthetase and glutaminase. Glutamine synthetase catalysis involves the acyl-phosphate γ-glutamyl-P; (b) exogenous glutamine can serve as both a nitrogen and carbon source; sequential action of glutaminase and glutamate dehydrogenase can liberate both amide and amino nitrogens as ammonia, while transaminases that utilize glutamate as amino donor move the amino group onto many ketoacid frameworks. The deaminated α-ketoglutarate scaffold can serve as TCA cycle substrate and as cofactor for the oxygenases noted in Figures 15 and 16.

(2) in pyrimidine biosynthesis. CAP is a mixed anhydride between carbamic acid and phosphoric acid (Figure 9A), termed an acyl phosphate, and has the characteristic thermodynamic activation of such anhydrides for group transfer of either the phosphoryl group or the carbamoyl group to cosubstrate nucleophiles. It may be that such acyl phosphate anhydride structures were early inventions of thermodynamically activated scaffolds that could last long enough in aqueous media to begin to power steps in metabolic pathways. They tie back intimately to ATP as a generating metabolite.

In analogy to acetyl-CoA as a doubly reactive metabolite, so are acyl phosphates. As mixed anhydrides, they are subject to capture by nucleophiles, both at the electrophilic acyl carbonyl group or at the elctrophilic phosphorus atom. The regiochemistry of acyl versus phosphoryl transfers to cosubstrate nucleophiles are determined by the specific enzyme catalysts, as noted in several examples below.

Two biosynthetic enzymes, carbamoyl phosphate synthetases I and II, that utilize carbamoyl-P as donor function in two quite different arms of nitrogen metabolism but use the same group transfer chemical logic.[87,88] Both have glutaminase domains that hydrolyze Gln to Glu and NH_3 to provide the nascent ammonia required for reaction with carbonic acid to generate sufficient carbamic acid in the active site to attack the γ-phosphoryl group of cosubstrate Mg-ATP (Figure 9A). The two synthetases also sit in different cellular compartments, cytoplasmic for the synthetase that funnels carbamoyl groups into RNA and DNA building blocks, versus mitochondrial for the synthetase that channels excess nitrogen into urea as an excretable end metabolite. The two synthetases are also regulated by different activator and inhibitor small molecules to modulate nitrogen flux anabolically and catabolically.[89−92]

In terms of chemical logic for one carbon unit activation, two related acyl phosphates are also metabolites that fit the reactivity pattern (Figure 9b). One is the acyl phosphate of formic acid, formyl phosphate.[93] A second is the mixed anhydride of carbonic acid and phosphoric acid, termed carboxy phosphate (Figure 9b) that is an intermediate in the conversion of acetyl-CoA to malonyl-CoA at the start of fatty acid biosynthesis. (It undergoes phosphoryl transfer in the back direction and carbonic acid transfer in the front direction of biotinyl enzymes). Both these acyl-Ps are also formed from ATP as intermediates in formylation and carboxylation steps in purine biogenesis. Two additional acyl-Ps, glycyl-P and CAIR-P, are likewise generated from ATP in two additional steps of purine biosynthesis (Figure 9b). Thus, this acyl-P logic is utilized four successive times to generate acyl groups as

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electrophilic fragments in construction of two of the four bases in RNA and DNA.

The related two carbon acyl phosphate, acetyl-phosphate, is produced by bacterial acetate kinase and then reacted with CoASH via phosphotransacetylase catalysis to produce the hydrolytically more stable acetyl-CoA. All these acyl phosphate metabolites arise by enzyme-directed attack of a carboxylate oxygen on the electrophilic γ-P group of Mg-ATP, releasing Mg-ADP and the mixed anhydride product. The enzyme acetyl-CoA synthetase imposes a different regiospecificity on the acetate carboxylate such that it is positioned to attack the α-P of ATP to generate acetyl-AMP. Acyl phosphates are on the border of sufficient kinetic stability to be useful diffusible, primary metabolites in cells while acyl-AMPs seem to have given way to acyl thioesters, with no loss in thermodynamic group transfer potential while gaining aqueous kinetic stability.

Perhaps one might have anticipated cells would utilize carbamoyl-S-CoA as a preferred carbamoyl donor rather than carbamoyl-P but that path is not evident in contemporary organisms. This thermodynamically activated acyl phosphate has just enough kinetic stability to be able to diffuse to and be used by enzymes for carbamoyl transfers ($t_{1/2}$ at physiological pH and 37 °C is ~5 min).[94] Analogously as noted earlier, in the energy-generating and capture steps of glycolysis, glyceraldehyde-3-P dehydrogenase (GAPDH) generates a 3-phosphoglyceryl-*S*-enzyme thermodynamically activated thioester and then releases it via attack from inorganic phosphate to produce 1,3-diphosphoglycerate with a newly formed acyl phosphate linkage. That acyl-P is harvested in the next enzymatic step by 3-phosphoglycerate kinase, converting ADP to ATP by phosphoryl transfer from the mixed anhydride.

5.2. Carbamoyl Transferases

Aspartate carbamoyltransferase (aspartate to *N*-carbamoyl aspartate) *N*-acylates the amino group of aspartate in the first committed step of pyrimidine biosynthesis that leads on to uridine and cytidine RNA and DNA building blocks (Figure 9a). The second enzyme example, ornithine carbamoyltransferase, has a more pedestrian but still essential cellular function, in the urea cycle, to enable safe excretion of excess nitrogen in an unreactive, nonucleophilic form (Figure 9a). The chemistry in each case is attack by a nucleophilic amino group (Asp or Orn) on the activated carbamoyl carbonyl to form a tetrahedral intermediate which can decompose in the front directon with loss of phosphate.[95] The accumulation of the *N*-carbamoylated end products is driven by the favorable thermodynamics of capture of the mixed carbamoyl-phosphoric anhydride on the carbonyl group by the cosubstrate amines.

5.3. Glutamine, Glutamate, ATP, and the Management of NH_3 Levels

We noted in the prior section that management of NH_3/NH_4^+ levels is critical to the nitrogen cellular economy. This is a key concern given the potentially promiscuous behavior of the free NH_3 form as an unwanted nucleophile. Two ATP-utilizing routes are employed to capture free NH_3 (Figures 9a and 10a). We noted the action of the carbamoyl phosphate synthases (CAPS) in the previous section, which ultimately pass the NH_3 into pyrimidine scaffolds or excrete it as urea.

The second catalyst is glutamine synthetase, connecting acyl phosphate to amide formation and the logic of the previous section. In this enzyme active site, the γ-carboxylate of glutamate attacks the γ-P of ATP to produce γ-glutamyl-P as a transient intermediate (Figure 10a). Attack on this acyl phosphoric anhydride (on the carbonyl group not the phosphoryl group) by cosubstrate NH_3 generates the stable amide linkage in glutamine.[96] The product glutamine is then the diffusible metabolite that can release NH_3 in the active site of various glutaminases. From this perspective, one of the key attributes of glutamine is a "just in time" or latent source of ammonia in restricted microenvironments of specific enzyme active sites. To emphasize the convergent logic of ATP, acyl-Ps, glutamine, and nascent ammonia at another stage of purine biosyntheses, we note the enzymatic conversion of 5-phosphoribose-1-pyrophosphate (PRPP) to 5-P-ribosyl-1-amine at the start of purine biosynthesis.[26]

5.4. Glutamine as a Nitrogen and Carbon Source

Pluripotent stem cells need glucose and glutamine to survive under culture conditions.[97,98] Glucose functions as an energy source for aerobic glycolysis (noted above) with insufficient flux through the TCA cycle to power respiration. Glutamine is taken up by a dedicated cell membrane transporter and then imported via a transport protein into the mitochondria where it can sequentially give up its two nitrogens as ammonia, under the action first of glutaminase and then the NAD^+-dependent glutamate dehydrogenase (generating an equivalent of NADH in the process) (Figure 8 and 10b). Either the released NH_3 or the initial glutamine, acting as a latent carrier of ammonia, provide the nitrogen atoms for both amino acids and the building blocks for RNA and DNA synthesis.[78,99,100]

The α-ketoglutarate arising from the tandem action of glutaminase and glutamate dehydrogenase can feed into the TCA cycle at the level of the α-ketoglutarate dehydrogenase complex, bypassing the block in aconitase and isocitrate dehydrogenase. The second half of the TCA cycle (ketoglutarate to oxalacetate) removes six electrons (as three hydride equivalents), providing 2 NADH and one $FADH_2$, capable of yielding 6.5 ATPs through coupling to the respiratory chain. This route of glutamine catabolism has been observed in many cancer cell lines growing in culture.[101,102] Moreover, glutamine is a significant carbon source in small intestine and kidney metabolic flux.[103] For ammonia generation in the kidney during starvation, glutamine is converted to α-ketoglutarate, whose carbons can subsequently be used for gluconeogenesis in concert with ammoniagenesis. However, it is not clear that glutamine is an equivalent major carbon source for tumors in vivo, as opposed to in culture dishes.[78,99,104]

An alternative metabolic fate exists for the mitochondrial glutamate, available from glutaminase hydrolytic action.[97] Rather than be oxidized by glutamate dehydrogenase to the imino-glutamate that then hydrolyzes to ammonia and α-ketoglutarate, glutamate can be transaminated directly to α-ketoglutarate while oxaloacetate and pyruvate can be reductively aminated to aspartate and alanine by their respective transaminases (Figure 10b). This route avoids release of free NH_3 by directly transferring the amino moiety between keto acid frameworks.

A brief aside about the transaminases found in mitochondria and cytoplasm of many cell types. All those enzymes utilize the aldehyde form of vitamin B_6, pyridoxal-P (PLP), as tightly bound coenzyme to move the amino group between α-keto acid backbones. Indeed, PLP is the signature coenzyme for almost every metabolic transamination, decarboxylation, epimerization, and aldol cleavage in the manipulation of amino groups during biosynthesis of amino acids.[73] In its tight binding to the apo forms of its constituent enzymes, PLP

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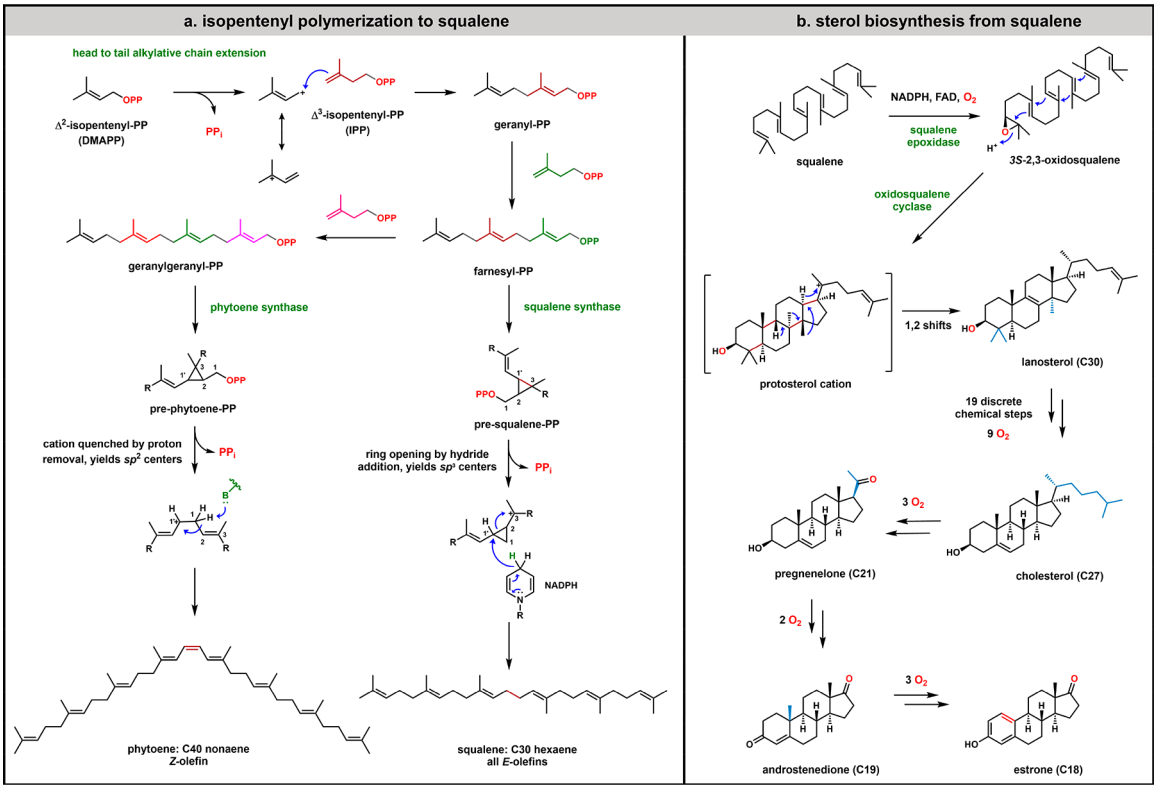

Figure 11. Isoprenoid metabolism: (a) isopentenyl-PP regioisomers are the key building blocks for the C_{30} hexaene squalene and the C_{40} nonaene phytoene. Chain elongations go by C_5 head to tail elongations via iterative ally cation chemistry up to the C_{15} and C_{20} stages before C−C bond formation switches to head to head C_1 to C_1 connections in squalene and phytoene, with cyclopropyl cation formations and rearrangements involved in presqualene and prephytoene formation and breakdown; (b) sterol biosynthesis from squalene involves prior epoxidation of the 2,3-double bond, a cyclization cascade to create the familiar tetracyclic framework, and a series of 1,2 H and CH_3 migrations before release of the C_{30} lanosterol. A series of oxygenases then act in different tissues, on unactivated carbon sites, to convert the C_{30} lanosterol to C_{27} cholesterol to C_{21} pregnenelone to C_{19} androstenedione. The cytochrome P450 enzyme aromatase carves out the angular A/B ring methyl group of androstenedione to aromatize the A ring as male sex hormone is converted to the C_{18} female sex hormone estrone.

has functional analogies to the FMN and FAD coenzymes noted in an earlier section. By virtue of the high affinity specific protein binding constants, neither flavins nor PLP are appreciably free in cells, for good reasons. The dihydroflavins would be futilely autoxidized in fractions of seconds, while the aldehyde group of PLP would be adventitiously reactive for condensation with any amine-containing metabolite.[12,39]

Glutamine is also the primary nitrogen donor in proliferating cells.[105] As cells exit quiescence and reenter the cell cycle, they have increased energy needs and nitrogen requirements to gear up for growth and proliferation. Glutamate dehydrogenase, glutamine synthetase, glutaminase, and the cadre of transaminases move the amino group nitrogen atoms through anabolic and catabolic pathways that incorporate (nucleotides, RNA, DNA, and protein biosynthesis) or excrete them, while cooperating to generate enough α-ketoglutarate to run the TCA cycle in times of need.

5.5. Redox Currency for Nitrogen Metabolism

NAD(H) and NADP(H) both play a key role as hydride donors/hydride acceptors in the reversible interconversion of glutamate and α-ketoglutarate as some glutamate dehydrogenases can use one form or the other of the nicotinamides as electron currency. The ready reversibility in cellulo of glutamate dehydrogenase enables this enzyme to balance flux between tricarboxylate cycle and energy metabolism versus amino acid metabolic pathways. Glutamate and glutamine levels can reach millimolar concentration or higher, depending on the cell type,[105−108] some 5−10 fold higher than other proteinogenic amino acids, in keeping with their roles as nodal point metabolites.

6. ISOPENTENYL-PP ISOMERS AND HEAD TO TAIL PRENYL TRANSFERS

6.1. Molecular Basis of the Utility of Isopentenyl-PP

Section 2.3 noted how the C_5 framework of Δ^3-isopentenyl-PP arises from tandem condensation of three acetyl-CoA molecules by the initial enzymes of isoprenoid pathways. The next enzyme in the pathway isomerizes the double bond from the 3- to the 2-position, generating useful quantities of both the original Δ^3-isomer and the Δ^2-IPP (Figure 11a). Each of these regioisomeric IPPs has a central and complementary role in building up isoprenoid frameworks.[9]

The Δ^2-isomer is a ready source of a five-carbon allyl cation equivalent through C_1−OPP bond cleavage at enzyme active sites. The C−O bond cleaves with low energy because the resultant C_1 carbocation is stabilized by delocalization of the positive charge at C_3, the definition of a resonance-stabilized allyl cation. In turn, the Δ^3-IPP can offer up the π electrons of its double bond as nucleophile to attack the allyl cation. The result is a head to tail alkylation event: alkylation of the terminal carbon of the Δ^3-IPP by the allyl cation derived from the Δ^2-IPP. Loss of a proton to quench the nascent product cation yields the C_{10} product geranyl-PP that is specifically a Δ^2-

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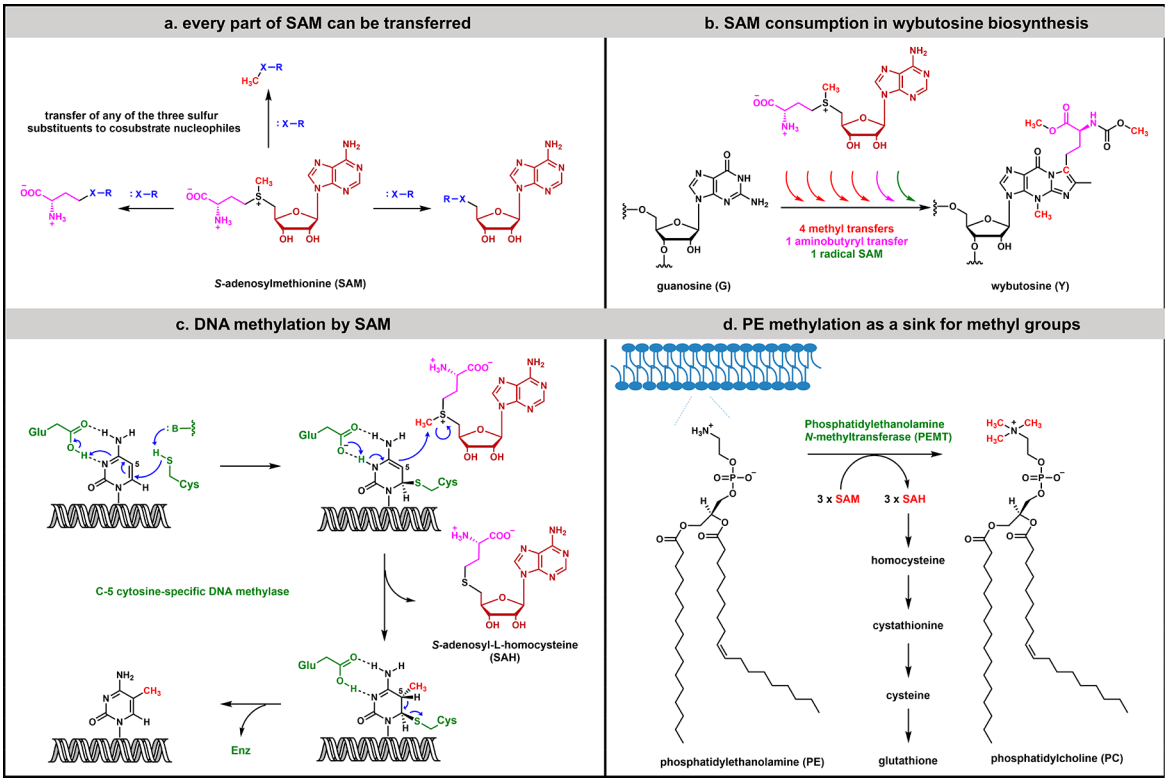

Figure 12. *S*-adenosyl methionine (SAM) as thermodynamically activated alkyl transfer metabolite: (a) the sulfonium group in SAM activates all three substituents for transfer as electrophilic alkyl fragments, although methyl group transfer is by far the most common; (b) the versatility of SAM is evident in formation of the wybutosine-modified base in tRNA. Six equivalents of SAM are consumed, four are moved as $[CH_3^+]$ equivalents, the fifth as a $[CH_3\bullet]$ equivalent. The sixth molecule of SAM instead donates an electrophilic aminobutyryl group. (c) DNA methylation at C_5 of cytidine residues occurs by the indicated addition/elimination mechanism with SAM as the one carbon donor. (d) SAM is also involved in membrane phospholipid maturation, converting hundreds of thousands to millions of phosphatidylethanolamine to phosphatidylcholine molecules via three consecutive *N*-methyl transfers in mammalian cells.

prenyl-PP (Figure 11a). So it can function as the source of an electrophilic C_{10} carbocation in a subsequent round with the Δ^3-IPP.

We note explicitly that the carbon nucleophile used by prenyl transferases is a terminal C═C double bond, specifically the π electrons. Double bonds are used only rarely as carbon nucleophiles in metabolism, another notable case being the formation of cyclopropane fatty acids from Δ^9 olefins with SAM (vide infra) as the C_1 (CH_2) donor.[109]

A second head-to-tail chain elongation by a five-carbon unit generates the C_{15} farnesyl-PP (Figure 11a). This type of five carbon head-to-tail chain elongation process can be repeated in cells to accumulate different length prenyl-PP products, some with *Z*-olefin (*cis*) geometries in place of the *E*-geometries (*trans*) shown. Thus, seven elongations would generate a C_{40} species (for electron transfer quinones), 10 would yield the C_{55} bactoprenol-PP in bacterial cell wall assembly, and 21 elongations would yield the C_{110} dolichol-PP that is the oligosaccharide carrier in *N*-glycoprotein assembly.[110]

The Δ^2- and Δ^3-IPP isomers are building blocks for some 50000 known isoprenoid metabolites, most of them isolated historically from plant sources.[9] As such, they may be the most widely used building blocks in all of metabolism. It is the Δ^2-IPP isomer specifically that is the facile donor of the electrophilic five carbon prenyl group. In contrast to acetyl-CoA which is the premier biological acyl donor, Δ^2-IPP and longer congeners such as farnesyl-PP and geranylgeranyl-PP are alkyl donors. They can undergo both head-to-tail and as noted below, head-to-head alkylations, and can be captured by other nucleophiles, including thiol side chains of cysteine residues in proteins.[111]

6.2. Farnesyl-PP and Geranyl-PP: Head-to-Head Prenyl Transfers

One of the most remarkable attributes of some enzymes that act on the C_{15} and C_{20} length Δ^2-prenyl-PPs (farnesyl- and geranylgeranyl-PP) is their ability to catalyze head-to-head rather than head-to-tail self-alkylations (Figure 11a). Squalene synthase converts two molecules of farnesyl-PP to the linear C_{30} hexaenoic hydrocarbon squalene.[112] Correspondingly, phytoene synthase in plants converts two molecules of geranylgeranyl-PP to the C_{40} olefin phytoene, which has nine double bonds.[113] The mechanisms of these head-to-head conversions have been worked out and shown to proceed through cyclopropane-containing intermediates that rearrange to the C_1−C_1 coupled products (Figure 11a) (summarized in ref 9).

Although beyond the scope of this presentation, this is another manifestation of how the allyl- and rearranged carbocation reaction manifolds open up novel metabolic pathway options by producing rearranged scaffolds. After generating squalene, organisms then go on to morph it from a linear hexaene to the tetracyclic framework of sterols. Animals epoxidize squalene at the 2,3-double bond and then cyclize it to lanosterol (Figure 11b). Plants instead make variant polycyclic scaffolds, including β-amyrin.[114] Thousands of steroid variants have been found between plant and animal kingdoms, reflecting

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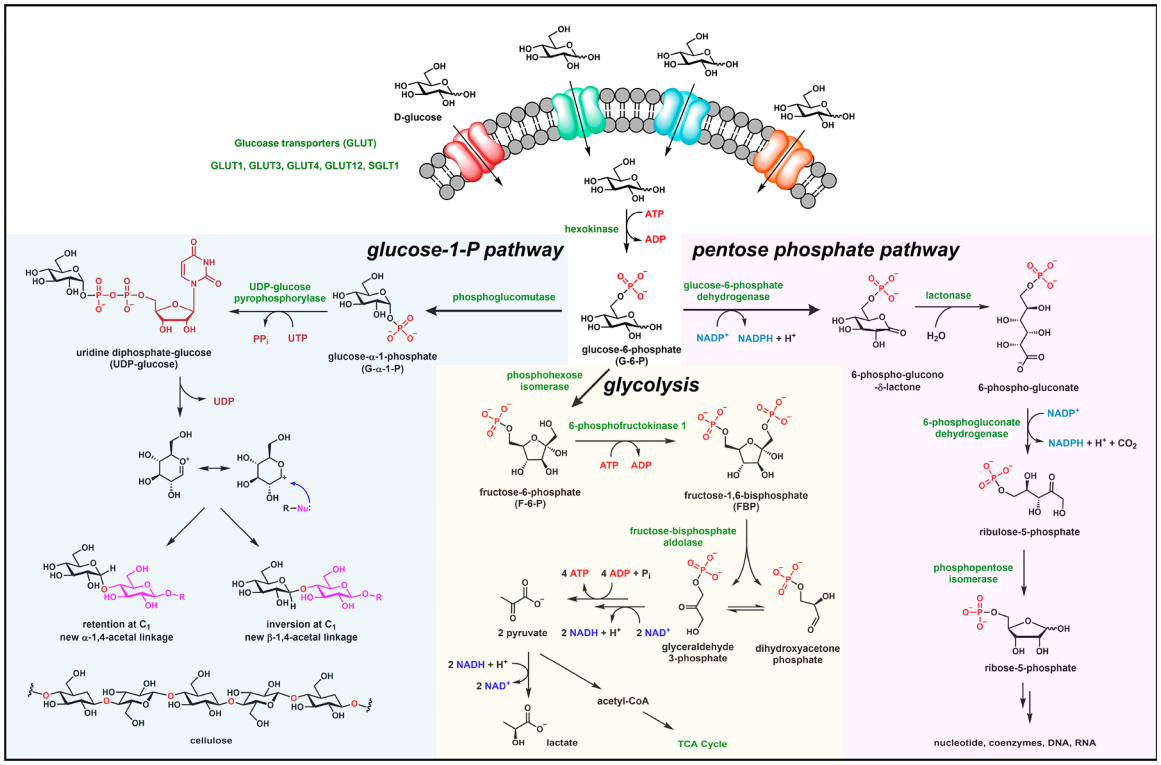

Figure 13. Three main pathways for cellular glucose utilization: in addition to the glycolysis and pentose phosphate pathways for flux of glucose-6-P (refer also back to Figure 3), the third pathway branches off from glucose-1-P, via action of phosphoglucomutase. The resultant UDP-glucose is the proximal donor of glucosyl moieties as glucosyl C_1 oxocarbenium ion equivalents in oligosaccharide, polysaccharide, and glycoprotein biosynthesis, resulting in C_1 connectivity of all such transferred glycosyl moieties. Gluconeogenesis is not included in this figure.

both differential quenching of sequential cationic intermediates in the cyclization process and extensive oxygenative maturation steps.

7. *S*-ADENOSYL METHIONINE: SULFONIUM ION ACTIVATED FOR GROUP TRANSFERS

S-Adenosylmethionine (SAM) may be the most widely used metabolite/coenzyme for group transfer reactions in cells after ATP. It is formed from ATP and L-methionine by an unusual attack of the methionine sulfur, at the oxidation state of sulfide, on the $C_{5'}$ carbon of the ribose ring of ATP, a net adenosyl transfer (Figure 4d).[115] During the reaction, the nascent triphosphate is hydrolyzed to PP_i and P_i before release, reflecting a 10^5 coupling in the direction of SAM accumulation. SAM is unusual in containing a positively charged trivalent sulfur, a sulfonium ion group. This is the structural basis of its thermodynamic activation as a group transfer agent. On the other hand, the concentration of SAM in cells, in the range of 10 μM, is a minute percentage (~1−3%) of the ATP concentration,[116] reflecting diversion of a small fraction of the ATP pool. The kinetic stability is adequate for its biologic roles, but nonenzymatic epimerization at the trivalent sulfur will produce a biologically inactive SAM diastereomer[117] and it can fragment nonenzymatically to methylthioadenosine and homoserine lactone. At a nominal concentration of about 2×10^7 molecules of SAM/human cell and its high utilization for the kinds of alkyl transfers elaborated below, there is a premium on metabolic regeneration of the SAM metabolic pool (e.g., from *S*-adenosyhomocysteine following methyl transfer) to ensure sufficient capacity for its multiple roles in small molecule and protein metabolism.

In principle, any of the three substituents at the sulfur of SAM are activated for group transfer to a cosubstrate nucleophile, with consequent quenching of the positive charge on sulfur as it reverts to a divalent state (Figure 12a).[109] As depicted in Figure 12b, one can observe transfer of the aminobutyryl group in formation of the modified tRNA base wybutosine[118] or aminopropyl transfer to putrescine after SAM decarboxylase action, yielding the C_7 triamine spermidine. One can also observe transfer of the ribose substituent in formation of queuosine, another modified base in tRNA.[119,120]

However, far and away it is the transfer of the one carbon methyl substituent to cosubstrate nucleophiles that defines SAM in cellular metabolism. All three of the bond cleavages in Figure 12a are alkyl transfers, with the methyl transfer a C_1 group transfer. It is of interest to compare the chemical logic and enzymatic machinery of these C_1 alkyl transfers versus the C_5 alkyl transfers from isopentenyl-PP above. Each involves an electron deficient carbon species: one carbon or five carbon alkyl group in flight but the chemical nature of the activation in the starting material SAM versus Δ^2-IPP is dramatically different.

Almost all of the perhaps tens to hundreds of millions of methyl transfers per cell cycle involve transfer of a $[CH_3^+]$ equivalent to a nucleophilic oxygen, nitrogen, sulfur, or carbon atom. In the wybutosine example above, four such methyl transfers have occurred (Figure 12b).[9] Methyl transfers in neurotransmitter metabolism include *N*-methylation of noradrenaline to adrenaline and deactivating catechol to *O*-methyl catechol transformations. Methyl transfers to C_5 of cytosine residues in DNA involve net C-methylation of an isoamide

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carbon nucleophile in an enzyme-DNA covalent adduct intermediate (Figure 12c).[121]

Among the most-studied methyl transfers in recent decades have been the suite of lysine side chain methylations in histone tails in chromatin (Figure 18a).[122,123] Strictly speaking, this is SAM acting as substrate for protein posttranslational modifications rather than primary small molecule metabolism, but these are quantitatively important in terms of SAM consumption.[124] There may be 500 million histones per human cell and up to 10 methyl groups on Lys side chains of H3 and H4 at steady state, emphasizing the large flux of methyl transfers down this pathway.[125] Along with the orthogonal histone acetylations noted earlier, these methyl marks are thought to signal differing states of transcriptionally active chromatin. Here is a case where metabolomics of SAM affects transcriptomics, then proteomics, and presumably a broader reach of metabolomics throughout the cell. One recent study has indicated in *Drosophila* that increased activity of glycine *N*-methyltransferase, generating sarcosine, extends lifespan.[126] It is unlikely this is an effect of rising sarcosine levels; rather it is a net decrease in SAM concentration. SAM-dependent methylations in other post-translational modifications of proteins are further examined in section 11.

A second metabolic pathway that is a significant sink for methyl group transfers from cellular SAM is the phospholipid biosynthetic enzyme carrying out the first of three *N*-methylation steps as phosphatidylethanolamine (PE) is converted to phosphatidylcholine (PC), both bulk phospholipid components of cell membranes (Figure 12d).[127] Studies have suggested the histone methylations and membrane PE methylations may be important components/sinks in regulation of cellular fluxes of SAM to the reaction product *S*-adenosylhomocysteine to facilitate *trans*-sulfuration and the subsequent synthesis of cysteine and glutathione from methionine and SAM.[127] The amount of PE in *E. coli* cells is estimated around 2×10^7 molecules.[13] In a eukaryotic cell with a thousand-fold larger volume, if there would be 10^9 to 10^{10} PE molecules and 30% undergo 1−3 methylations to yield phosphatidylcholine, this could be a massive drain on the SAM concentrations in cells.

8. GLUCOSE-6-P, GLUCOSE-1-P, AND UDP-GLUCOSE

As glucose enters cells, the first chemical event is ATP-dependent phosphorylation of glucose at C_6, driven by the 10^5 equilibrium kick as ATP is fragmented to ADP so essentially all glucose molecules are trapped as the impermeant phosphomonoesters. Three metabolic fates await glucose-6-phosphate (glucose-6-P) (Figure 13).

8.1. Glycolysis: Three ATP-Dependent Sugar Kinases

Glucose-6-P can be processed down the ten enzyme glycolytic pathway and then the TCA cycle noted above as the key energy-generating route in cell metabolism. The hexokinase/glucokinase reaction is effectively irreversible under all physiologic conditions. The ATP-dependent conversion of fructose-6-P to the fructose-1,6-diphosphate and ADP is also effectively irreversible under physiologic conditions. The third kinase in glycolysis, pyruvate kinase, actually runs in the ATP-generating direction from phosphoenolpyruvate but can be reversed under glucoeneogenesis conditions in hepatocytes[128] and other tissues.

8.2. Pentose-P Pathway: NADPH Generation

A second route for flux of glucose-6-P is the pentose phosphate pathway, with the $NADP^+$-specific gatekeeper enzyme oxidizing C_1 by two electrons to the 6-P-gluconolactone scaffold and NADPH.[41] Hydrolytic ring opening by a lactonase presents the 6-P-gluconate for a second $NADP^+$-reducing oxidoreductase. This enzyme is similar in logic and mechanism to the oxidation/decarboxylation path of isocitrate dehydrogenase in the TCA cycle. Oxidation of the 3-OH of 6-P-gluconate to the ketone, with reduction of $NADP^+$ to NADPH transiently generates the 3-keto-6-P-gluconate which, as a β-keto acid, undergoes facile decarboxylation via the intermediate enolate to D-ribulose-5-P. This is an enzymatic isomerization away from D-ribose-5-P (Figure 13), the crucial building block for nucleotide biosynthesis. These five carbon phospho sugars give the pentose-P pathway its name.

The 5-P-ribulose and 5-P-ribose, keto and aldehyde sugar isomers, respectively, can be processed by transketolases and transaldolases to transfer C_2 and C_3 fragments, respectively, to other sugar scaffolds to yield C_4 to C_6 scaffolds. Among these is the C_4 erythrose-4-P, a central building block in plants and microbes that have the genes and enzymes for the shikimate pathway to phenylalanine, tyrosine, and a plethora of other shikimate-derived aromatic metabolites.

Conversion of glucose-6-P to ribulose-5-P and CO_2 in the first three enzymatic steps of the pentose phosphate pathway constitutes a four-electron oxidation of the glucose framework. These four electrons, transferred as two hydride equivalents, are stored in two molecules of NADPH. This energy capture is the main route to cytoplasmic NADPH which we have noted above is the preferred donor of hydride ion in fatty acid biosynthesis, in HMG-CoA reduction, in glutathione reduction, and as detailed in a subsequent section, in two electron donation to cytochrome P450-dependent oxygenases that tailor sterol scaffolds in steroid hormone and vitamin D maturations.

8.3. Glucose-1-P Pathway: UDP-Glucose as the Activated Glucosyl Donor

The third potential significant flux of glucose carbon in anabolic pathways starts with its enzymatic isomerization by phosphoglucomutase to the α-isomer of glucose-1-P (Figure 13). We note below that this glucose-1-P stereoisomer is the substrate for attack on UTP to give UDP-glucose. Thus, ATP, NADPH, and UTP/UDP-glucose are intimately involved in directing the metabolic fluxes of intracellular glucose.

This seventh group transfer strategy is responsible for driving assembly of all intracellular and extracellular oligosaccharide, polysaccharides, lipopolysaccharides, proteoglycans, and *N*- and *O*-linked glycoproteins.[9] The key building blocks are nucleoside diphospho sugars where the glycosyl residues are activated for capture at C_1 by any of a large number of cellular nucleophiles. UDP-glucose is the paradigmatic activated building block in mammalian cells, but ADP-glucose is the monomer for starch in plants, reflecting utilization of both pyrimidine and purine triphosphates as glycosyl donors (GDP-mannose and CDP-paratose are additional examples of metabolic sugar donors, indicating all four common nucleoside triphosphates in cells are ultimate energy sources for sugar-based biopolymer assembly).

Focusing on UDP-glucose as prime example, UTP is attacked at α-P by one of the phosphate oxygens of glucose-α-1-P in a nucleotidyl transfer mediated by the gatekeeper enzyme UDP-glucose pyrophosphorylase/UTP-glucose-1-phosphate uridylyltransferase to produce UDP-glucose and PP_i (Figure 13). The

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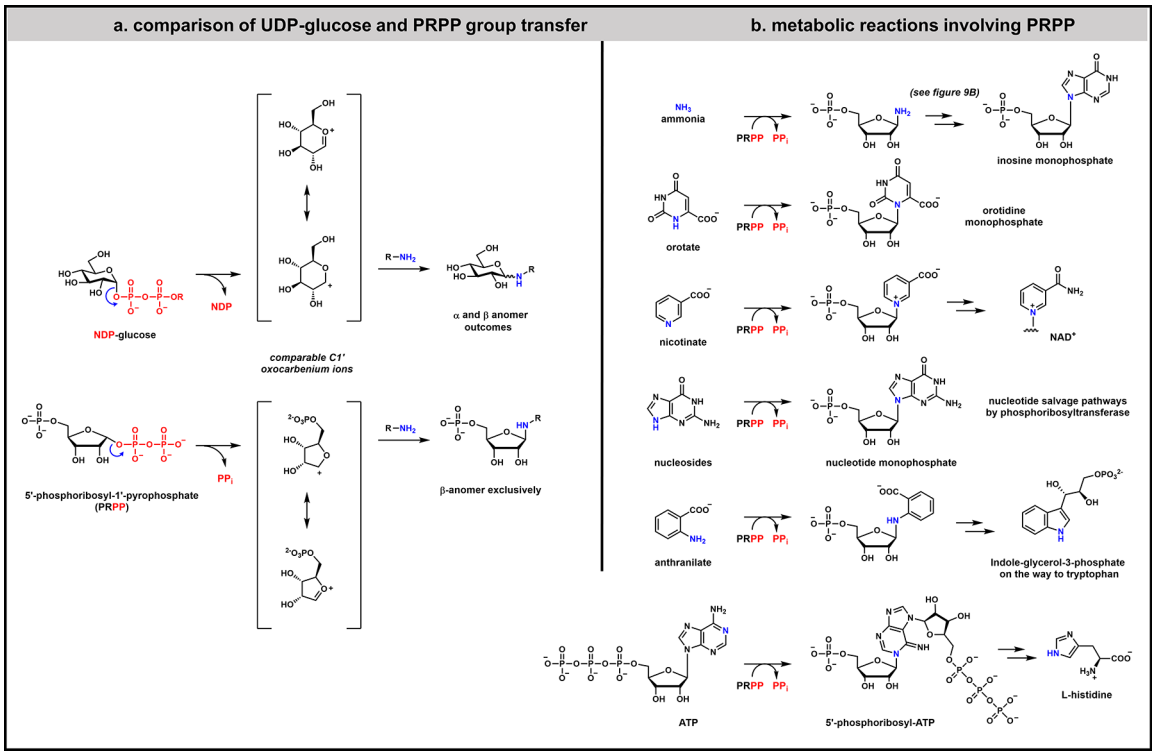

Figure 14. 5-Phosphoribose-1-pyrophosphate (PRPP) as activated phosphoribosyl donor (a) UDP-glucose and PRPP are metabolites activated by the C1-pyrophosphate groups for C1−O bond cleavage via oxocarbenium ion transition states; (b) PRPP serves as an electrophilic phosphoribosyl donor to a set of cellular amine cosubstrates, including de novo purine and pyrimidine nucleotide biosynthesis, purine, and pyrimidine salvage, NAD^+ biosynthesis, and also to provide some of the carbon atoms in both tryptophan and histidine biogenesis.

cleavage of a pyrophosphoric anhydride bond between α-P and β-P indicates expenditure of energy, expressed as a push on the equilibrium in the forward direction. The resultant UDP-glucose is activated for glucosyl transfer at C_1. UDP-glucose is likely synthesized in the cytoplasm and then can be transported into the lumen of the endoplasmic reticulum,[129] consistent with the *O*- and *N*-glycosylation of proteins transiting through the intracellular secretory vesicle systems.

Inspection of the potential transition state with early C_1−O bond breakage indicates that the developing positive charge at C_1 is delocalized into the oxygen bridging to C_5, an oxocarbenium ion that is kinetically accessible in the active sites of thousands of glycosyl transferases. Capture at C_1, for example by the oxygen atom at C_4 of a glucosyl moiety from an elongating starch chain can occur from the front side to give glucosyl-α-1,4-glucose$_n$ or from the back side to a growing cellulose chain (β-1,4-glucose$_n$) to give glucosyl-β-1,4-glucose$_n$, depending on whether starch synthase or cellulose synthase is the catalyst (Figure 13). Iteration of these transfers hundreds of times or more builds up starch, glycogen, and cellulose, as three examples of polysaccharides with energy storage or mechanical functions for cells.

In a different set of metabolic intersections, the assembly of the tetradecasaccharyl-dolchol as the starting point for all *N*-glycoprotein glycosylation events uses equivalent logic via glycosyl transferase action on NDP-hexose building blocks attached to the long chain isoprenyl dolichol-P.[130] The universal reliance on NDP-hexoses as glycosyl group donors to a wide range of cosubstrate nucleophiles ensures that essentially every glycosyl residue in biology is regiospecifically connected to its neighboring O, N, S, or C atom through C_1.

It is worth noting that the ADP-ribosyl transfers from NAD^+ and the glycosyl transfers from UDP-glucose have a similar underlying chemical logic from the point of view of the groups undergoing transfer. In each transition state an electron-deficient C_1 oxocarbenium ion (glucosyl vs ADP ribosyl) is attacked by a nucleophilic cosusbstrate atom to achieve glycosylation of the nucleophile. From the point of view of the leaving groups, one is UDP, the other is nicotinamide, the strategies are distinct and presumably represent convergent evolution to arrive at thermodynamically activated glucosyl/ribosyl moieties embedded in the kinetically stable scaffolds of NAD^+ and UDP glucose.

8.4. 5-Phosphoribose-1-pyrophosphate (PRPP) as Activated Phosphoribosyl Donor

While glucose is the most central hexose in metabolism, D-ribose is the corresponding pentose, given it is the core scaffold in the nucleotides that make up all RNA species and, after conversion to the 2′-deoxyribose at the deoxynucleotide diphosphate level, the corresponding scaffold in DNAs. We noted above in section 8.2 that the pentose-P pathway generates 5-P-ribose from glucose-6-P, but it is the 5-P-ribose-1-PP (PRPP) that is the functional ortholog to UDP-glucose.

As noted in Figure 14A, both UDP-glucose and PRPP have sugars bearing a pyrophosphate group at C_1 that functions as a low energy leaving group. PRPP like UDP-glucose is both thermodynamically activated (K_{eq} for hydrolysis ~3500)[26] and sufficiently stable kinetically to function in this case as a phosphoribosylating agent for a set of cellular amine nucleophiles. While UDP-glucose is assembled by nucleotidyl transfer to glucose-1-P, PRPP is generated enzymatically by a

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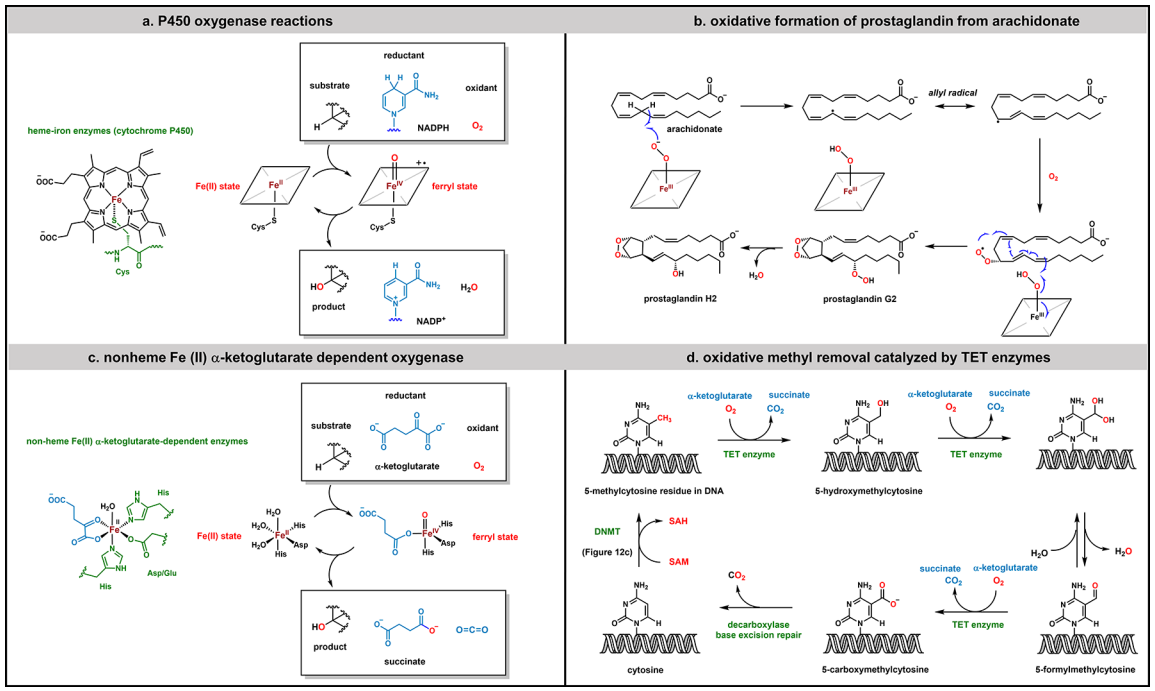

Figure 15. Two main types of iron-based monooxygenases: (a) heme-based cytochromes P450 use high valent ferryl oxygen intermediates as strong oxidants for one electron chemistry to effect C−H bond homolysis at unactivated carbon sites and OH• rebound for net hydroxylation; (b) the maturation of arachidonate into PGG2 and PGH2 involves an initial allyl radical conversion to a pair of peroxy radicals one of whch closes to the cyclopentane endoperoxide characteristic of PGG2 and PGH2; (c) the typical two His, one Glu/Asp coordination set for nonheme mononuclear iron oxygenases also includes cosubstrate α-ketoglutarate as the 4th and 5th ligands to Fe. An analogous Fe^{IV}═O high valent oxoiron species performs comparable homolytic C−H bond cleavage and OH• equivalent transfer; (d) oxidative demethylation of 5-MeC residues in DNA is mediated by the TET subfamily of nonheme mononuclear iron oxygenases, in three sequential steps as shown.

rare pyrophosphokinase (PRPP synthetase, see section 2.3) catalyzing attack of the C_1−OH of the α-anomer of 5-P-ribose on β-P of Mg-ATP (Figure 4C). One might have anticipated that UDP-ribose would have been the UDP-glucose equivalent, and indeed UDP-ribose is a known microbial metabolite for ribosyl transfer in antibiotic biosynthetic pathways.[9] It may be that evolution to PRPP was an early event that allowed generation of membrane impermeant anionic nucleotide monophosphates rather than neutral nucleosides that could have diffused out of cells.

In contrast to UDP-glucose and related NDP-hexoses that function largely in oligosaccharide chain elongations, PRPP is used predominantly in nitrogen metabolic pathways. These include de novo purine and pyrimidine biosynthesis, salvage pathways for all free nucleobases, an early step in NAD^+ biosynthesis, and in the production of both histidine and tryptophan (Figure 14b).[26] In all these cases, one can formulate the mechanism as an early cleavage of the C_1−OPP linkage to an oxocarbenium ionlike transition state, akin to that featured for UDP-glucose (Figure 14a). All amine captures result in inversion of stereochemistry at C_1 of the P-ribosyl moiety, generating β stereochemistry.

In de novo purine biosynthesis, the amine nucleophile is nascent NH_3, generated in the enzyme active site by controlled hydrolysis of glutamine, to yield P-ribosyl-1-amine as the first committed step in the pathway (Figure 9b). By contrast, in pyrimidine assembly, we noted the condensation of carbamoyl-P and aspartate as the starting reaction (Figure 9a). Subsequently, N_1 of orotate is the nucleophile attacking PRPP to yield the nucleotide orotidine-5-P, a decarboxylation away from UMP. This is also the reaction type for all salvage reactions to scavenge free nucleobases at N_1 and return them to nucleotide monophosphates.

One of the routes to NAD^+ involves nicotinate coupling to PRPP as shown in Figure 14b, with the pyridine nitrogen as the nucleophile. The pathways to histidine and tryptophan are among the more complex of the proteinogenic amino acids (Figure 14b). The start of the histidine pathway involves the rare attack of N_3 of ATP on C_1 of PRPP, while tryptophan biosynthesis involves *N*-phosphoribosylation of anthranilate before opening of the ribose on the way to indole glycerol-P. Clearly PRPP provides a crucial link between ribose and nitrogen metabolism.

9. O_2: THERMODYNAMICALLY ACTIVATED, KINETICALLY STABLE INORGANIC MOLECULE TO POWER EUKARYOTIC METABOLISM

9.1. Realizing the Thermodynamic Benefit of O_2 as Terminal Electron Acceptor: From TCA Cycle to Cytochrome Oxidase

The respiratory chain membrane proteins embedded in the inner mitochondrial membrane are iron- and copper-based redox components that mediate the transfer of electrons one at a time through a series of controlled potential drops, finally to the terminal redox protein cytochrome oxidase (Figure 3).[73] Cytochrome oxidase, with two heme iron atoms and two copper atoms can store four electrons and then transfer them to O_2 molecules, one at a time without loss of any of the formal intermediates (superoxide, peroxide, hydroxyl radical), to reduce them to two molecules of water each. Coenzyme Q quinones are lipid soluble mobile 2/1 step-down electron transfer molecules that work upstream of the B and C

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cytochrome subtypes. Each of the cytochromes in the respiratory chain has a different Fe^3 to Fe^2 one-electron reduction potential based on variations in the heme macrocycle and/or the apoprotein environment. This enables the heme proteins to function as way stations ordered by relative alignments and their redox potentials. The cytochrome-filled respiratory chain is a one-electron conduit all the way to O_2 (Figure 3).

NADH transfers electrons to a 4-subunit protein mitochondrial membrane complex known historically as complex I[131] that acts functionally as an NADH dehydrogenase (Figure 3). Electrons flow two at a time as a hydride ion to bound FMN and then one at a time to iron−sulfur clusters before connecting to the cytochrome gauntlet. Correspondingly, the reduced form (E-$FADH_2$) of succinate dehydrogenase, generated during TCA cycle, feeds electrons into the multiprotein complex II way stations in the mitochondrial membrane respiratory chain.

Compared to the $E^{o\prime}$ for electron transfer in the NADH/NAD^+ couple of −340 mV under physiologic conditions and $FAD/FADH_2$ of −210 mV (although tight protein binding can move the midpoint of the potentials), various cytochrome midpoint potentials (Fe^3 to Fe^2) range from −100 to +500 mV for the terminal cytochrome oxidase while the reduction potential for the O_2 to $2H_2O$ conversion is +0.82 V.[10] Electrons passing from NAD(P)H to O_2 thus fall through a potential drop of (0.34 + 0.82) = 1.16 V, indicating a $K_{eq} \sim 10^{38}$ for oxidation of NADH and reduction of O_2 enabled by the respiratory chain components (Figure 2b). This is one measure that O_2 reduction is immensely thermodynamically favored, provided kinetic barriers to one-electron transfer steps can be overcome.

9.2. Iron-Based Oxygenases Reductively Activate O_2 To Oxygenate Lipophilic Metabolites

It is estimated that ∼95% of reductive dioxygen metabolism in higher eukaryotes is via the mitochondrial respiratory chains noted above. The remaining 5% of O_2 metabolism is again reductive, by a net two electrons in oxidase mode or by a net four electrons in the oxygenase mode.[9,39] O_2 is a substrate for hundreds of oxygenases that act in secondary metabolic pathways in microbes and plants to build complex natural product scaffolds.[9] Those oxygenases often tailor hydrophobic nascent scaffolds to build in both functional group chemistry and to provide hydrophilic/hydrophobic balance to the product framework. Perhaps this logic is best exemplified in maturation of taxadiene into taxol, the microtubule-disrupting antitumor drug. The C_{20} hydrocarbon framework of taxadiene undergoes eight oxygenations on the way to taxol.[9]

In contrast, few of the primary metabolic pathways in higher eukaryotes, including humans, employ oxygenase-mediated chemistry extensively. While flavin-based oxidases are used in some amine oxidase pathways,[39] the great bulk of the mammalian oxygenases are iron-dependent, generating high valent oxoiron intermediates, in both heme (Figure 15a) and nonheme frameworks (Figure 15b), to carry out radical-based oxygenations of cosubstrates. A suite of heme-containing cytochrome P450 family oxygenases work on steroid frameworks as noted below. Two related sets of nonheme iron oxygenases carry out consequential oxygenative demethylations of histones and DNA and are noted in section 11, subsequently.

9.2.1. Steroid Oxygenations: Lanosterol to Estrone: Oxygenative Metabolism at Its Most Extensive. Of particular note are the conversions of the initial C_{30} tetracyclic framework of lanosterol on to C_{27} cholesterol, then the set of C_{21} adrenal hormones, and finally the C_{19} androgen and C_{18} estrogen frameworks of the sex hormones (Figure 11b). From lanosterol to cholesterol, three angular methyl groups are carved out oxidatively by monooxygenases which collectively activate and reduce nine molecules of O_2 in that process.

Cholesterol in adrenal glands is subject to removal of the seven carbon isovaleryl side chain by action of three cytochrome P450 oxygenases. Then a cytochrome P450 type of $C_{17,20}$ lyase removes the residual acetyl group and converts the C_{21} steroid to a C_{19} androgen androstenedione. Finally, the remarkable enzyme aromatase oxygenatively removes the angular C methyl group of the A ring, aromatizing it to give the C_{18} estradiol. From lanosterol to estradiol, 12 carbons are carved out of the initial tetracyclic framework via action of some 6 oxygenases reducing 17 molecules of O_2 (Figure 11b).

We have noted the thermodynamic reduction potential of O_2 in an earlier section. In the monooxygenase reactions summarized here, NADPH is the typical electron donor to bound FAD or FMN in the oxygenase multienzyme complex. The FAD acts in its unique mode as a 2/1 step-down electron switch, accepting a hydride two electron packet from NADPH and then reoxidizing by one electron paths ($FADH_2$ to FADH• to FAD) to feed electrons one at a time to iron centers, either directly to the cytochrome cofactor of the P450 oxygenases or via intermediacy of an iron−sulfur cluster electron transfer protein.

9.2.2. Vitamin D and Prostaglandin Oxygenative Metabolism. The metabolic precursor to vitamin D is 7-dehydrocholesterol which undergoes a sequential set of rearrangements to give the vitamin D framework. Sequential vitamin D3 hydroxylations by cytochrome P450 monooxygenases in two different tissues, liver and kidney, produce the trihydroxy end metabolite calcitriol. These transformations can be viewed as part of the chemical logic of hydroxylation of steroid family members to achieve distinctive biological functions.

A third example of heme protein-based oxygenation occurs in the arachidonate to prostaglandin pathway of lipid hormones, involving formation of allylic hydroperoxides and cyclic endoperoxides, effected by a pair of cyclooxygenase isozymes (Figure 15b). The oxygenative modifications of steroid and fatty acid substrate frameworks are late stage events in the respective pathways and may represent recent evolutionary additions to the metabolic repertoires in contrast to oxygen-independent core metabolic transformations.

9.3. Mononuclear Nonheme Iron Oxygenases for Methyl Cytosine Demethylation

The second major family of iron-based monooxygenases do not use a heme cofactor but instead rely on two His and one Asp or Glu side chain carboxylate as ligands to the active site $Fe^{(II)}$ (Figure 15c). Typically this oxygenase superfamily requires α-ketoglutarate as oxidizable cosubstrate to generate the high valent $Fe^{(IV)}$=O active oxygen transfer species. Removal of the CH_3 group from 5-Me-cytosine residues in DNA occurs oxygenatively, by a family of TET nonheme mononuclear oxygenases (Figure 15d).[132,133] The mechanism is akin to the oxygenative demethylation of Me-Lys side chains in histones summarized in section 11. Cytidine demethylation in DNA occurs after egg fertilization, in embryogenesis, and in some cancer cells.

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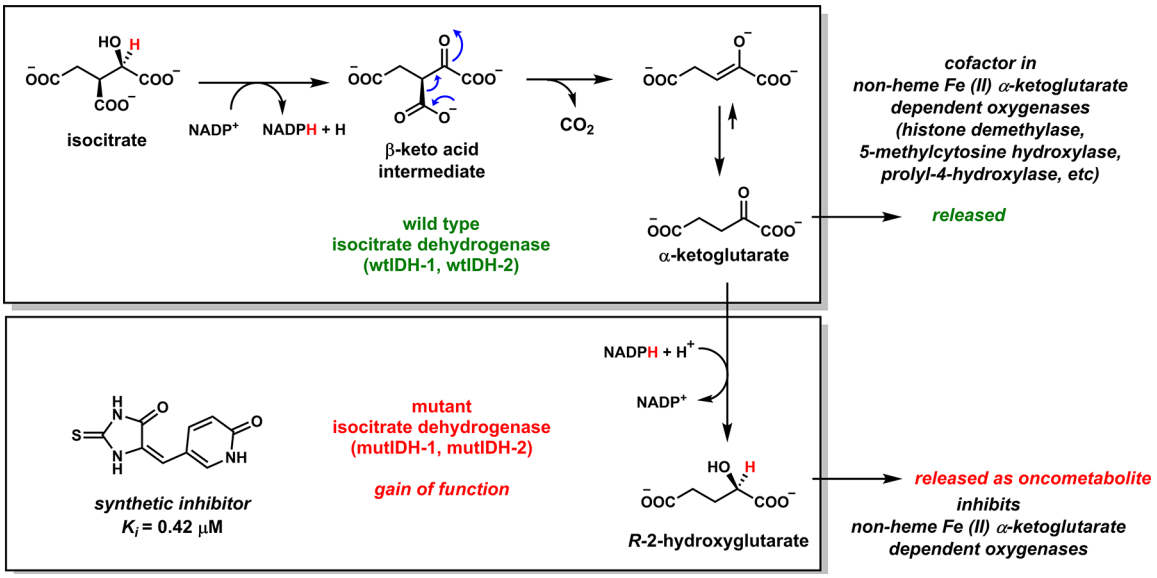

Figure 16. Product profile from wild-type and mutant form of isocitrate dehydrogenase (IDH-1): isocitrate is oxidized to the oxalosuccinate β-keto acid that is then subjected to actve site-mediated regiospecific decarboxylation of one of the three carboxylates to yield α-ketoglutarate. In addition to its role in the TCA cycle, α-KG is the oxidizable cosubstrate for nonheme mononuclear iron monoxygenases, some of which are shown in the top panel (also see Figure 14). In the mutIDH-1 catalytic cycle, product release is slowed long enough for the nascent α-KG to be reduced by the incipient NADPH product to yield *R*-2-hydroxyglutarate. This alternate product is an inhibitor of the mononuclear iron oxygenases by competition at the α-KG binding site and has been termed an oncometabolite.

The initial product from 5-methylcytosine residues is the corresponding 5-hydroxymethylcytosine residue. Unlike the *N*-CH_2OH nascent products from histone *N*-methyl oxygenations which are spontaneously hydrolytically labile, the C−CH_2OH linkage is chemically stable. Thus, net dealkylation of cytosine residues in DNA requires two more sequential TET family oxygenation events (logic akin to the sequential triple oxygenations in lanosterol C_4 and C_{14} demethylations in section 9.2.1), via 5-formyl-cytosine and 5-carboxy-cytosine products before ultimate loss of CO_2 (Figure 15d).[134]

In all monooxygenases, the substrate undergoing hydroxylation is oxidized by two electrons while the O_2 molecule is reduced by four electrons (Figure 15). Thus, there always needs to be a second substrate that gives up two electrons for the reaction to balance. For all the P450 oxygenases, that cosubstrate is NADPH (less frequently NADH), tying NADPH electron currency availability to these biosynthetic oxygenations (Figure 15a). For the nonheme Jumonji and TET family oxygenases, the cosubstrate undergoing co-oxidation is not NADPH but instead α-ketoglutarate, a TCA cycle intermediate that allows regulation of the two types of iron-based oxygenases by metabolic state (Figure 15c). As will be noted below, the mutant forms of isocitrate dehydrogenase lead to *R*-2-hydroxyglutarate alternate product molecules that can shut down the α-ketoglutarate-dependent histone and DNA demethylases.

It is possible that this sequestration of oxygenase chemistry into the restricted sets of pathways, notably in steroid hormone maturation and on macromolecular protein and DNA substrates, reflects late evolution of these catalytic capacities. All the other primary metabolic pathways (glycolysis, TCA cycle, pentose phosphate pathway, fatty acid synthesis, purine and pyrimidine biosynthesis, protein and nucleic acid macromolecular syntheses) had arisen in anaerobic organisms before planetary oxygenation levels rose. Second, it may be that one should think of steroidogenesis, vitamin D maturation, and carotene and vitamin A formation as secondary metabolism, not primary metabolism in that same evolutionary sense.[135]

9.4. From IDH-1 Mutations to Blockade of Histone and DNA Oxygenative Demethylases

Perhaps the most significant example that has refocused attention on perturbations in primary metabolism that are relevant to human diseases (e.g., in gliomas, acute myeloid leukemias, and other tumors) are the mutations in the one of the three isoenzyme forms of isocitrate dehydrogenase, IDH-1.[136] IDH-1 is cytoplasmic, while IDH-2 and IDH-3 are mitochondrial; the latter functions in the TCA cycle. IDH-1 and IDH-2 use $NADP^+$ as a cosubstrate,[137] while IDH-3, the classic tricarboxylate cycle participant, uses NAD^+ and generates NADH as product. Possible functions for cytoplasmic IDH-1 are to provide α-ketoglutarate as cofactor for the class of oxygenases noted above that function to oxygenatively demethylate N-Me_{1-3}−Lys histone tails in chromatin and also to hydroxylate 5-methylcytosine residues in DNA,[136] and to supply an additional source of NADPH.

The mutations in IDH-1 (R132H, R132C) that are oncogenic reroute the flux of isocitrate such that the nascent α-ketoglutarate and NADPH products are not released as usual but instead held long enough for the bound NADPH to reduce the ketone to *R*-2-hydroxyglutarate (Figure 16).[138] This alternative hydroxyglutarate product, as opposed to normal α-ketoglutarate product, can rise to 100-fold higher concentrations in glioma versus normal cells.[136,138] The hydroxyglutarate can then act as a competitive inhibitor of α-ketoglutarate, with particular import in blockade of α-ketoglutarate-dependent mononuclear, nonheme iron oxygenases in the histone demethylase class, and the oxygenative conversion of 5-methylcytosines in DNA for loss as CO_2. It is now clear that 2-hydroxyglutarate is produced even in normal cells, especially under hypoxia.[139,140] IDH mutants produce *R*-2-hydroxyglutarate while *S*-2-hydroxyglutarate can come from action of L-lactate dehydrogenase and malate dehydrogenase on α-

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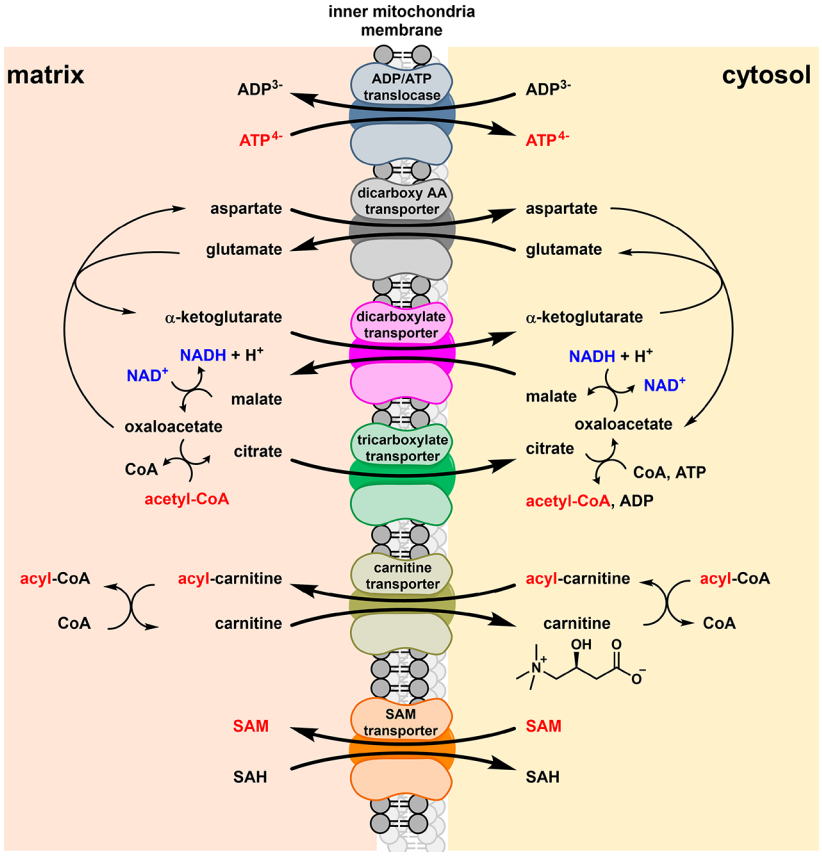

Figure 17. Schematic of six inner mitochondrial membrane transporters for ATP/ADP, aspartate/glutamate, α-ketoglutarate/malate, citrate, acyl-carnitine/carnitine, and SAM/SAH.

ketoglutarate. Both 2-HG isomers can act as inhibitors of α-ketoglutarate-dependent oxygenases.

2-Hydroxyglutarate therefore qualifies as an oncometabolite.[136] Blockade of these demethylases leads to elevated levels of methylated histones and aberrant regulation of gene transcription that can promote unrestrained cell growth. This novel metabolite can be detected at low levels by mass spectrometry, in principle allowing classification of tumors with IDH mutations.[141] Medicinal chemistry programs are underway that have defined inhibitors specific to the mutant forms of IDH-1[142,143] and would have promise as antitumor agents in such disease states. The IDH-1 mutation story links altered NAD(P)H redox metabolism to reductive activation of O_2 and SAM modification of proteins and DNA.

Molecular oxygen is a wild card metabolite. Higher eukaryotes unlock its thermodynamic potential to undergo four electron reduction and make a good living energetically. They practice substrate hydroxylation chemistry judiciously, in hypoxia, in macromolecule demethylations, and in the steroid hormone maturation pathways. Yet, they still have had to evolve enzymatic and nonenzymatic defenses against toxic partially reduced oxygen metabolites, emphasizing how oxygen reductive metabolism has its intrinsic dangers.

The dioxygen molecule joins the other seven thermodynamically activated molecules of this chapter as companion molecule that can power multiple metabolic steps. A gaseous, inorganic, biogenerated metabolite, O_2 offers itself as a dramatic energy sink to organisms that can feed in electrons one at a time. The mechanistic incompatibility between two electron-only NADH and one electron-only O_2 reactivity could be viewed as the main boundary condition for aerobic life and an energy tie-in between glycolysis, the pentose phosphate pathway, the TCA cycle, and the respiratory chain components.

10. SPATIAL INTEGRATION OF METABOLISM: POOLS OF KEY METABOLITES

Most metabolic pathways are compartmentalized within cells, although some have steps with involvement of more than one organelle and a rare subset, such as vitamin D maturation, involve both kidney and liver oxygenative metabolism to get to the final active calcitriol metabolite.[135] For passage of metabolites between cytoplasm and nucleus [e.g., SAM, acetyl-CoA, or ATP to enable chromatin methylation and acetylation and for DNA replication and RNA transcription (to name just a few nuclear events)], it is likely that nuclear pores are sufficiently large to permit passive diffusion of these low molecular weight central metabolites.

On the other hand, diffusional passage of charged molecules between mitochondria and cytoplasm does not occur and has required the expression and membrane insertion of families of transport proteins. The most well-studied group may be the ATP-ADP translocases (Figure 17) which allow export of mitochondrial ATP down its concentration gradient to the cytoplasm (we have noted 5 mitochondrial NADHs generated for each cytoplasmic NADH during glycolysis).[144] In turn, ADP is countertransported from cytoplasm to mitochondria, required for continuing synthesis of ATP during respiration.

The amount of ATP in a 70 kg human has been estimated at ~50 g, with about 10^9 molecules/cell,[20] most of it generated in mitochondria. The turnover of ATP molecules by the two types

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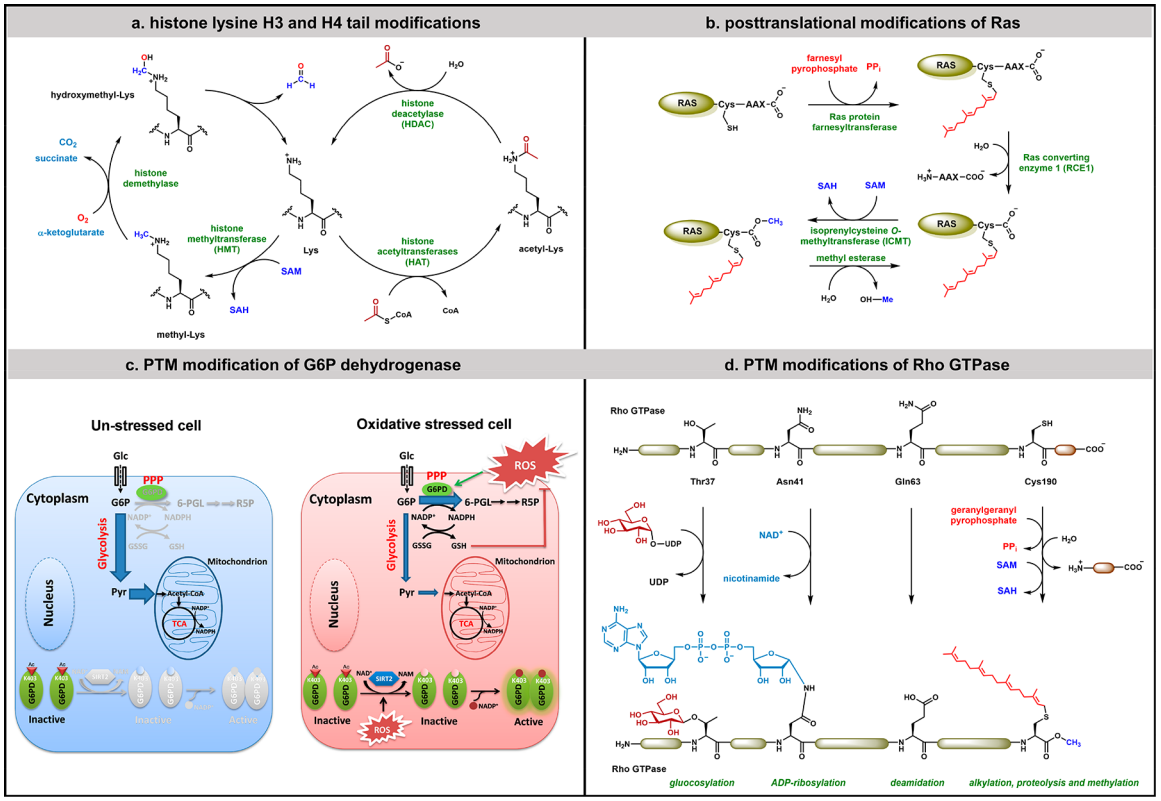

Figure 18. Seven central metabolites that power small molecule metabolism also dominate post-translational modification of proteins: (a) the two most common histone lysine H_3 and H_4 tail modifications involve acetyl-CoA for acetylations and SAM for methylations. (b) The small GTPase family member Ras is processed at the C-terminus for alkylation by a C_{15} Δ^2-prenyl-PP (farnesyl-PP). This sets up proteolytic processing by Ras converting enzyme 1. The third post-translational modification is a SAM-dependent carboxy-*O*-methyltransferase. (c) Glucose-6-P dehydrogenase, the gatekeeper enzyme to the pentose phosphate pathway, undergoes interconversion between acetylated inactive forms and deacetylated active forms, where the deacetylase is the NAD^+-dependent sirtuin SIRT2. Reprinted with permission from ref 161. Copyright 2014 John Wiley and Sons. (d) Another small GTPase family member Rho undergoes a series of PTMs that use three of the key seven metabolites: UDP-glucose to glucosylate the side chain −OH of Thr37, ADP ribosylation from $NADP^+$ on the side chain amide of Asn41, and carboxy-*O*-methylation of Cys190 from SAM after prenylation and proteolysis to reveal Cys190 as the new C-terminus.

of side chain cleavages has been suggested to be ~0.3 s^{-1}. Kornberg suggested that up to 80 kg of ATP is cycled every day, indicating both dramatic utilization of ATP to power metabolism and a need for robust transport of ATP out and ADP into mitochondria. Most cells have nucleoside diphosphokinase enzymes that convert UDP, CDP, GDP back to the nucleotide triphosphates as ATP is converted to ADP. In turn, adenylate kinase will take two ADPs to ATP and AMP reversibly for equilibration of mono-, di-, and triphosphate pools.

Mitochondrial membranes typically also have monocarboxylate transporters, dicarboxylate transporters, and tricarboxylate transporters (Figure 17), the latter moving citrate back and forth.[73] The passage of citrate by this transporter is one device for moving acetyl units back and forth between mitochondrial and cytoplasmic CoA-SH stores. Mitochondrial acetyl-CoA, generated either from pyruvate dehydrogenase action or fatty acid β-oxidation enzymes, can react with oxaloacetate, as noted in an earlier section under agency of the first enzyme in the TCA cycle, citrate synthase. After catalyzed hydrolysis of nascent citryl-CoA to citrate and free Co-ASH to ensure accumulation of the tricarboxylate, the citrate can be carried across the mitochondrial membranes to the cytoplasm by the tricarboxylate transporter.

There it can be cleaved by the enzyme ATP citrate lyase into oxaloacetate and acetyl-CoA as ATP is cleaved to ADP and P_i.[145] The ATP cleavage pattern is consistent with logic that yields citryl phosphate as early intermediate, then the more kinetically stable citryl-CoA, before retroaldol cleavage to yield acetyl-CoA (Figure 7b). The ATP cosubstrate is used to produce the thermodynamically activated acetyl thioester product. These acetyl-CoA molecules in the cytoplasm then undergo carboxylation to malonyl-CoA and are funneled into fatty acid biosynthesis. Acetyl groups can also move between mitochondria and cytoplasm by transfer of the acetyl group to carnitine, passage of acetyl carnitine via its transporter, and recapture of the acetyl group by cytoplasmic or mitochondrial CoASH.[146]

Moving redox equivalents of NADH between mitochondria and cytoplasm is also indirect. It can involve glycerol-3-P transporters in adipose tissue or malate shuttling via dicarboxylate transporters in liver and muscle cells.[147] The transport of malate from cytoplasm to mitochondria is functionally equivalent to the transport of a hydride to NAD^+ in different cell compartments. Cytoplasmic oxaloacetate (e.g., from ATP citrate lyase action, vide supra) is reduced to L-malate by malate dehydrogenase using cytoplasmic NADH as a reducing agent. Malate is transported selectively into mitochondria and then oxidized by mitochondrial malate dehydrogenease (enzyme six of the TCA cycle) back to oxaloacetate as NAD^+ is reduced to NADH. De facto, this is the equivalent of moving two electrons into the mitochondrial pool

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Chem. Rev. 2018, 118, 1460−1494

of NAD^+. Mitochondrial oxaloacetate is not a solute for the malate transporter. It can be transaminated to L-aspartate which is a substrate for a dicarboxy amino acid transporter. Once in the cytoplasm, L-Asp is transaminated back to oxaloacetate (Figure 17).

One further example involves transfer of SAM between nucleus (DNA, RNA, and chromatin protein methylations), cytoplasm, and mitochondria (biotin and lipoamide assembly in acetyl-CoA carboxylase and the lipoamide acyl transferase components of pyruvate dehydrogenase and ketoglutarate dehydrogenase complexes).[120] Passage between nucleus and cytoplasm is perhaps via free diffusion, while it is now clear that there is a transporter that moves SAM into mitochondria and the demethylated SAH product back out into the cytoplasm for hydrolysis,[148] to keep SAH at concentrations below its inhibition constant (Figure 17). These four examples emphasize the value of interorganelle shuttles, and the range of strategies cells have developed to make these central group transfer metabolites available in the subcellular locales needed for specific pathways. Their interorganelle passage allows integration of the moment-to-moment needs for the seven kinds of reactions that these molecules engage in for their particular group transfers and displacement of otherwise unfavorable equilibria.

11. INTERSECTION AND CONVERGENCE OF THE POST-TRANSLATIONAL MODIFICATION UNIVERSE WITH SMALL MOLECULE METABOLISM

In one canonical view, metabolism is generally concerned with the conversion of low molecular weight molecules in cells, with more than 2000 such metabolites identified to date. On the other hand, much of the ATP spent in anabolic pathways is to build macromolecules: replication of DNA, transcription of RNA, and ribosomal assembly of thousands of proteins in any cell cycle. Admittedly, these are assembled from low molecular weight metabolites noted earlier as the essential building blocks that get incorporated into linear condensed biopolymers.

The subset of proteins with catalytic activity (i.e., enzymes) are the proximal agents responsible for metabolism in the sense that they carry out the directed flux of the low molecular weight metabolites into and through the multiple pathways in different organelles of the cell. Although there are only ~20000 genes in the human genome, there may be millions of protein variants, some from RNA splicing but most from post-translational modification (PTM) of protein side chains.[12] The many PTMs control/modulate protein localization, protein catalytic activity, and substrate recognition, and protein lifetimes among other features. The same key six group transfer metabolites (absent carbamoyl-P) we have highlighted as central players in driving metabolic pathways of low molecular weight molecules are the ones used most frequently in protein PTMs (Figure 18).[12,149] These protein modifications occur separately or in combinations.

The most common single PTM is phosphorylation of Ser, Thr, and Tyr side chains by ATP-dependent protein kinases. Tens- to hundreds of thousands of phospho variants of proteins may be formed transiently in human cells by the >500 members encoded in human kinomes. Many proteins can be phosphorylated at multiple residues, by single or multiple kinases. The fraction of a given protein subject to modification can depend on location within the cell (distinct pools) and the activity of the PTM enzymes. Hundreds to thousands of fractional molecular protein variants can be created and then returned to starting pools by protein phosphatases.[150] Just as the concentrations of low molecular weight metabolites can vary from moment-to-moment and be subject to readjustment, so too can the composition of post-translationally modified proteins.

The next sections detail a subset of the interplay of different varieties of PTMs from the central cast of thermodynamically activated low molecular weight group transfer donors.

11.1. Interplay between Acetyl-CoA and SAM in Histone Tail Modifications

The N_6 side chain amino group of Lys residues in the N-terminal tails of both Histone H3 and H4 in chromosomes are famously subject to either acetylation or competing methylation. Acetylation involves acetyl-CoA as donor via action of a set of histone acetyltransferases while the histone methyltransferase families use SAM as donor of one, two, or three methyl groups at some of the same lysine residues. The potential combinatorial patterns are numerous and allow for titration of transcriptional activity across chromosome regions. Replacement of acetyl groups by methyl groups requires prior removal of the acetyl moiety as acetate by families of hydrolytic histone deacetylases as well as NAD^+-dependent sirtuins. By contrast replacement of methyl groups on lysine side chains is oxygenative not hydrolytic, as noted above in the mononuclear oxygenase section, reflecting the differential chemical lability of *N*-acyl versus *N*-alkyl groups. The one carbon methyl group is removed as formaldehyde, a reactive and potentially toxic one carbon metabolite, as shown in Figure 18a. The fractional stoichiometry of acetylations and methylations on H3 and H4 can be substantial given perhaps 500 million histones per cell. Therefore, there is a large flux of acetyl and methyl groups committed to these PTMs.

11.2. Hypoxic Response

A metabolically notable set of oxygenations of a specific protein are at the heart of the hypoxic response of eukaryotic tissues. These are hydroxylations of Pro and Asn side chains on the hypoxia inducible factor HIF1α transcription factor protein subunit. We note explicitly that these hydroxyproline and hydroxyasparagine residues occur in the realm of post-translational modification of proteins rather than on classical small molecule metabolites discussed in section 9. Also these are catalyzed by mononuclear nonheme iron oxygenases, requiring α-ketoglutarate as an oxidizable cosubstrate.[9] Both the nonheme and heme-based oxygenases generate high valent oxoiron species as the radical-initiating catalysts and follow similar chemical logic in delivering one of the two oxygen atoms from O_2 to a formally unactivated carbon center on the substrate.

Using O_2-consuming oxygenases as the direct hypoxia sensors is an elegantly simple route to monitor $[O_2]$ in cells. At low pO_2, the oxygenases cannot run, the HIF subunit is not hydroxylated. In this state, it is not a substrate for a second post-translational modification, side chain lysine ubiquitylation, and so persists and can function as a transcription factor, activating hundreds of genes which set in motion the hypoxic response.[151] When HIF1α is hydroxylated posttranslationally, then it is a robust substrate for ubiquitylation and rapid degradation by the proteasome. The HIF1α subunit does not accumulate and so the hypoxia program does not run. It is tautologic that the HIF oxygenases would be a late stage

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Chem. Rev. 2018, 118, 1460−1494

acquisition once ambient O_2 levels had risen to sustain aerobic organism metabolism and the rich variety of protein post-translational modifications which carry much of the signaling flux and communication logic in cells.

11.2.1. Histone Demethylations are Oxygen-Consuming. A second set of oxygenases, again in the nonheme mononuclear iron superfamily as in the HIF example above, are the catalysts that carry out demethylation reactions of the lysine side chains of histone protein H3 and H4.[152]

The Jumonji family reductively activate O_2 at the expense of α-ketoglutarate oxidative decarboxylation to produce the Fe^{IV}=O actve site oxidant that can deliver an OH• equivalent to the methyl groups on Me_1, Me_2, and Me_3 forms of N-Me-Lys side chains in histones H3 and H4 (Figure 18a). Unlike acetyl groups of Lys side chains of histones, the *N*-methyl groups are not removable by hydrolysis. Only oxidative routes can undo the alkylations. The nascent *N*-hydroxymethyl products can then unravel spontaneously in aqueous solutions to the amine and an equivalent of formaldehyde (Figure 18A). Three such sequential oxygenations are required to take an *N*-Me_3-Lys side chain back to the Lys-NH_2 while generating three equivalents of formaldehyde. Given the abundance of histones in cells ($\sim 5 \times 10^6$ molecules) and a steady state methylation stoichiometry that could be anywhere from 1 to 10 methyl groups per nucleosome, the oxygenative demethylation capacity in cells may be highly robust. In passing, it is known that half-lives of histone acetylations are shorter than histone methylations,[79,153,154] perhaps correlating with the chemical ease of hydrolysis and a larger set of deacetylases.

11.3. Interplay between Prenyl Transfers and Methyl Transfers in Changing Cellular Address of Proteins as in the Small Family of Conditional GTPases

Proteins can also be alkylated with farnesyl or geranylgeranyl isoprenoid groups on cysteine thiolates. These C_{15} or C_{20} hydrophobic prenyl groups can target such proteins to membrane microenvironments.

One such protein class that undergoes these prenylations are the family of small GTPases, whose hydrolysis activity is both slow and conditional.[155] Among them are Ras and Rho subfamily GTPases which hydrolyze GTP in the service of some coupled cellular activity. The k_{cat} for GTP hydrolysis, can be less than 1 min^{-1}, and be accelerated by partner GTPase activating proteins (GAPs) and GDP exchange factor proteins (GEFs: release of GDP is typically rate-limiting in a catalytic cycle), reflecting the contextual conditionality of the presence of partner proteins and specific protein substrates (e.g., vesicle proteins). GTP hydrolysis is providing its usual thermodynamic role of driving coupled equilibria in the cell, in this family often for cellular work in cargo transport.[156]

A closer examination of the post-translational processing of proteins such as Ras and Rho isoforms reveals three distinct types of modifications (Figure 18b): (1) prenylation of one or more Cys thiolate atoms near the C-terminus; (2) proteolysis just downstream of the prenylated Cys residue and then (3) SAM-dependent methylation of the new C-terminus to create a methyl ester, quenching the carboxylate negative charge and further facilitating relocation of modified Ras from cytosol to membrane surfaces.[12] The availability of SAM and farnesyl-PP (Ras, Rho) and geranylgeranyl-PP (Rho) are both critical for full maturation of these small GTPases.

11.4. SAM and ATP as Interactive PTMs

A third example of interaction between two of the central group transfer metabolites in protein post-translational modifications occurs in a related *C*-terminal carboxylate methylation of the catalytic subunit of protein phosphatase 2A.[157−159] The catalytic subunit of PP2A assembles on a scaffold partner protein and can interact with regulatory B subunits that can affect subcellular localization and choice of specific P-Ser- and P-Thr-containing proteins as substrates. The activity of the PP2A catalytic subunit can be significantly altered by action of a leucine *C*-terminal methyltransferase which covers the *C*-terminal carboxylate charge in forming the *O*-methyl ester, the same chemistry as in Ras and Rho. This methyl substitutent can be removed hydrolytically, as methanol, by a partner carboxylesterase, enabling reversible control of PP2A activity.[160]

This SAM/ATP interaction logic is reprised in a recent case study on autophagy and growth control in yeast.[124] SAM inhibits autophagy and promotes cell growth in a manner dependent on the methyltransferase that modifies PP2A. The aforementioned carboxymethylation of PP2A was observed to be highly sensitive to intracellular methionine and SAM levels, thereby enabling PP2A to function as a metabolically regulated phosphatase. As such, protein phosphorylation status can be adjusted in tune with the availability of SAM. Thus, a tandem pair of PTM enzymes, guided by the availability of ATP and SAM as group transfer cosubstrates, are part of the sensory and chemical logic for control of survival responses in yeast and perhaps higher eukaryotic cells.

11.5. NAD^+ and Acetyl-CoA Opposing Regulation of Pentose Phosphate Pathway Flux by Posttranslational Modification of Glucose-6-P Dehydrogenase

A cogent example of how post-translational modification covalent chemistry can modulate flux through a primary metabolic pathway is provided by the regulation of glucose-6-P dehydrogenase activity. As noted earlier, this is the gatekeeper enzyme into the pentose phosphate pathway, controlling the major source of NADPH for reductive steps in fatty acid and sterol biosynthesis (e.g., HMG-CoA reductase and squalene synthase reactions) as well as ribose-5-P for nucleotide assembly and erythrose-4-P for aromatic amino acid formation.

When glycolytic flux is high in cells, the pentose-P pathway flux is often low, with little diversion of glucose-6-P.[161] A variety of mechanisms regulate G-6-P dehydrogenase activity, including subunit dimerization stabilized by binding of a structural $NADP^+$ molecule at the dimer interface. The side chain of lysine 403 is positioned at the dimer interface, and it can be covalently acetylated from acetyl-CoA in a post-translational acetyltransferase reaction (Figure 18c).[161] This covers the positive charge on the N_6 amino group and may also provide steric hindrance that prevents dimerization and leads to inactive dehydrogenase monomers. This is a molecular route to keeping the pentose-P pathway flux dialed down, with the reduction depending on the mole fraction of subunits acetylated.

Reversal of the Lys403 side chain acetylation is effected by the NAD^+-dependent Sir2 cytoplasmic deacetylase (Figure 18c), noted earlier in the section on nonredox functions of NAD^+. Sir2 activity is elevated in cells during oxidative stress and the increased activity leads to deacetylation and activation of the FOXOA3 transcription factor and also the increases in activity of G-6-P dehydrogenase. In turn, more NADPH is

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produced, powering glutathione reductase and the downstream glutathione peroxidase to destroy problematic peroxides. The use of Sir2 rather than an NAD^+-independent deacetylase links NAD^+, NADPH, acetyl-CoA levels and flux through the pentose-P pathway by balancing the post-translational acetylation state of the gatekeeper enzyme in the pathway.

11.6. Multiple Posttranslational Modifications of Rho GTPase: Utilization of ATP, NAD^+, UDP glucose, Geranylgeranyl-PP, SAM, and Indirectly Acetyl-CoA

The Rho subfamily of small GTPases can serve as the protein poster child for five of the six group transfer metabolites central jointly to metabolism and protein post-translational modifications.[162,163] In addition to the C-terminal geranylgeranylation (Cys_{190}) and O-methylation of the newly exposed C-terminal carboxylate (utilization of C_{20} prenyl-PP and SAM, as for Ras in Figure 18b above), Rho can suffer three other types of post-translational modifications. UDP-glucose can donate its glucosyl moiety to the side chain -OH of Thr_{37} with abrogation of GTPase activity (Figure 18d) (summarized in ref 12). NAD^+ can donate its ADP-ribosyl group to the side chain amide moiety of Asn_{41}.

The fifth modification, phosphorylation, can occur on Rho itself to target it for subsequent polyubiquitylation and destruction by the proteasome but also can involve self-phosphorylation of Rho-associated kinase by ATP.[163] Phosphoryl transfer controls lifetime of Rho as catalyst and this two-step process of phosphorylation and polyubiquitylation is a common motif for marking short-lived proteins in cells.

Finally, the sixth group transfer metabolite, acetyl-CoA, is used to acetylate the RhoA-specific GEF partner protein, completing the full panoply of group transfers and exemplifying extensive combinatorial diversification of proteomes.[164] The extent to which any given Rho protein molecule in a cellular population has multiple modifications is not well-quantitated nor is the net activity of such multiply modified Rho species well-evaluated.

11.7. Nonenzymatic Reactions of Thermodynamically Activated Metabolites?

One consequence of the combination of thermodynamic activation of the eight metabolites coupled to kinetic stability in physiologic conditions, is that they circulate in cells as diffusible intermediates with useful half-lives. This raises the possibility of unintended, nonenzymatic group transfers to cellular macromolecules leading to spurious covalent modifications.

In principle, any of the seven organic activated metabolites and the sole inorganic activated molecule O_2 could be susceptible to uncontrolled reactions. There is little evidence that ATP and congeneric NTPs, dNTPs, engage in non-enzymatic phosphorylations or nucleotidylations of proteins in phosphoproteomic analyses. Likewise, there seems to be little uncatalyzed liability to isopentenyl-PP as the alkylating agent. SAM, the premier biologic methylating reagent could transfer any of the three substituents on the sulfonium cation to cellular nucleophiles, but this potential reactivity does not appear to be notably problematic in cells. Nor are spurious glycosylations of proteins from NDP-sugars reported to be responsible for random glycosylations. (In contrast, hyperglycemic levels of blood glucose levels modify red cell hemoglobin to give the signature glycosylated hemoglobin A1C in diabetics.)[165]

Activated acyl groups are more of a problem. Of the three molecular species, acyl-AMPs, acyl-Ps, acyl-S-CoAs, all are equally activated thermodynamically. But, the acyl-AMPs and acyl-Ps are at much lower concentrations in cellular metabolism, and so nonenzymatic acylation/phosphorylations are likely to be of minor incidence and consequence. Not so for some of the predominant acyl-CoAs.

Acyl-CoAs such as acetyl-, malonyl-, and succinyl-CoAs are at higher local concentrations in cells. Thousands of proteins can be acetylated as detected by mass spectrometry, albeit the mole fraction and regiochemistry of such species are often uncertain.[166,167] Many proteins are also found to be succinylated, malonylated, propionylated, and crotonylated. It is possible that a majority of such acylated proteins form nonenzymatically, as whether there exist specific acyltranferases other than acetyltransferases is not clear.[64,168,169] Acylated proteins may be especially abundant in the mitochondria where there is thought to be a higher pH and concentration of acetyl-CoA and succinyl-CoA that could enhance rates of non-enzymatic acyl transfer,[170,171] most commonly to protein lysine residues. Consistent with this idea, several sirtuins are dedicated to the mitochondria as protein deacylases,[55,172−174] perhaps in a clean up role.

The eighth activated metabolite O_2 offers a distinct nonenzymatic liability from the above organic molecules. Nonenzymatic group transfer is not a problem. Rather it is adventitious one electron reduction to superoxide, for example in the presence of inorganic ferrous irons that can set off reactive oxygen species (ROS) cascades.[175] There is a vast literature on ROS chemistry and biology for oxidation of lipids, proteins, and nucleic acids, illustrating that O_2 is probably the most liable to nonenzymatic reactivity and cellular component modifications.

11.8. Convergent Chemical Logic and Reactivity

Many of the chemical modifications that occur up at the protein levels thus mirror the kinds of phosphorylations, acetylations, glycosyl transfers, acylations, and alkylations that occur down on the ground in primary metabolism. They may reflect an evolutionary expansion of the chemical reactivity built into this set of six group transfer molecules from low molecular weight modifications of metabolites to macromolecular modifications. The convergence of the same kinds of transformations emphasize a core chemical inventory and the utility of ATP, NADH, acetyl-CoA, SAM, IPP, and longer homologues, and UDP-glucose in both the low molecular weight and the parallel protein PTM metabolic universes. It is not yet clear how many kinases, acetyl transferases, or methyl transferases can carry out group transfers to both low molecular weight and protein substrates. Those that do so tend to have vastly different catalytic throughputs. On the other hand, the phosphoinositol kinases and microbial aminoglycoside kinases[176] are clearly related structurally and evolutionarily to protein kinase families. Regulation of intracellular location and activity of PTM-modified enzymes by the seven group transfer cellular reagents can clearly alter the small molecule metabolome.

12. CONCLUDING REMARKS

The systems biology approaches to the study of metabolism, under the rubric of metabolomics, have revived metabolism as a contemporary scientific activity. Interpretable connection of changes of metabolites to phenotypes is much more likely when perturbations in hundreds of metabolites can be measured simultaneously by mass spectrometry versus the classical approach of one metabolite at a time. Whatever the number

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of simultaneous measurements that can be made, it is helpful to have molecular insights into the logical framework that unites a small set of molecules that serve as nodal points for driving and integrating key pathways.

Of the eight small molecules highlighted in this review, all can be captured under the rubric of chemical stability in cellular conditions but readily activatable for thermodynamically favorable reactions by specific catalysts. Seven are organic molecules poised to carry out chemical work in metabolism, by displacement of coupled equilibria. The eighth, O_2, reacting by one electron reaction manifolds only, serves mostly as a thermodynamically favored electron sink but can also be reductively activated as oxygen transfer agent in late-evolving metabolic pathways.

ATP and NADH are central metabolic players in terms of energy harvesting and energy storage. Both can be accumulated in glycolysis as two molecules of pyruvate are produced, and this ancient pathway is independent of O_2. So is the tricarboxylate cycle where acetyl-CoA, the third prime-time energy-rich metabolite, is oxidized to two molecules of CO_2 and the eight electrons stored as three NADH and one $FADH_2$, available for driving dozens of coupled redox transformations. The intersection of phosphoryl group and acyl group chemistry is central in several primary metabolic pathways, exemplified by the diverse roles of carbamoyl-P in both anabolic and catabolic nitrogen metabolism.

When O_2 is introduced into the metabolic equations, the electrons that have been captured in NADH can flow via the one electron respiratory chain to carry out the remarkable four-electron reduction of O_2 to 2 H_2O with a 16-fold increase in ATP yield. This dramatic jump in ATP production is one compelling measure of the thermodynamic activation of O_2, achievable under the right chemical circumstances. The transfer of electrons from NADPH to O_2 via 2/1 step down flavin chemistry has enabled organisms to unlock the oxygenation potential of O_2 via high valent oxoiron species, to carry out dramatic oxygenations of unactivated carbon centers. This NADPH/O_2 interplay enables exquisite regiospecific conversion of hydrophobic to hydrophilic metabolite scaffolds and also provides the only obvious route to removal of methyl groups from proteins and DNA.

Inspection of the structures of NADH, the coenzyme A portion of acyl-CoAs, NDP-hexoses, and SAM reveal a common utilization of the AMP/NMP moiety over and over again as the structural (and perhaps evolutionary) foundation on which the specific group transfer moieties have become grafted. But the business end of the seven molecules is distinct and reveals that organisms have collected a customized set of organic (acyl phosphates, acyl thioesters, dihydropyridines, alkyl sulfoniums, allyl cation precursors, and glycosyl oxocarbenium ion precursors) and inorganic (phosphoric anhydrides, dioxygen) functional groups to carry out specific chemistries. These functional groups get spent on their own and in sequential, combinatorial action to power the primary metabolic pathways of life.

It is likely that most metabolomic studies of metabolite perturbations, be they genetic or pharmacologic, are reflecting the interconnected nodal points at which these seven central metabolites traffic in cells. The controlled reactivity of the group transfer molecules highlighted in this review spill over from small molecule metabolism to macromolecular decoration, in enzymes, glycoproteins, regulatory proteins, RNA, and DNA, emphasizing the logical and metabolic continuity of their use.

Gene content directly determines encoded protein content, via mRNA intermediacy, but the ~2000 (known) small molecule metabolites in cells and tissues are not directly encoded in DNA. To be sure the transformation of one set of metabolites into others is mediated by proteins with specific encoded catalytic activities and that includes the enzymes that build these seven group transfer metabolites. But the coalescence of catalytic machineries to stitch these small molecule scaffolds together to bring the palette of described chemistries to bear is a remarkable bottom up achievement to enable the chemistry of life, balancing the top down information in the genes themselves.

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